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**A BIOCHEMICAL HYPOTHESIS OF THE  
GENESIS OF CANCER\***

*By*

LOUIS A. PINCK

*Bethesda, Maryland*

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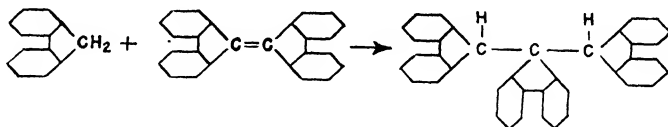
## A BIOCHEMICAL HYPOTHESIS OF THE GENESIS OF CANCER

Very little is known about the involved chemical mechanism of the genesis of cancer. In the words of Fieser,<sup>9</sup> "Considering particularly the evidence of the experiments in which examination was made of the whole animal and the excreta, it seems clear that the (carcinogenic) hydrocarbon suffers rapid alteration in the animal body. Some chemical reaction occurs and this apparently exhausts most of the carcinogen long before tumors begin to appear. Whether or not this reaction is involved directly or indirectly in the process of carcinogenesis, and whether changes in the tissue eventually leading to malignancy occur before or after the disappearance of the hydrocarbon, is still an open question. The most obvious hypothesis, based merely on a certain coincidence of two phenomena, is that the reaction in which the carcinogen disappears represents the first step in a time-consuming and complicated chain of events leading eventually to malignant growth."

In this article, an attempt is made to offer a working hypothesis of the mechanism involved in the set of phenomena leading to carcinogenesis which were outlined in the above statement of Fieser. It offers to explain why carcinogenesis is initiated by such exceedingly minute quantities of carcinogens, and, furthermore, it may account for metastasis. Since this hypothesis is based on a correlation of carcinogenic and chemical activities of carcinogenic compounds, it may, therefore, be possible to predict which compounds are potentially carcinogenic\* and which are not. Furthermore, the hypothesis may indicate a scientific method of approach in the study of chemotherapy in contrast to the present hit-or-miss procedure. It involves a rather complicated biochemical chain of reactions, which, once initiated, will be continuous.

Since the hypothesis is based on a novel type of Michael condensation first reported by Pinck and Hilbert, it is desirable to review this study.<sup>27, 28</sup> It has been shown that there are three types of Michael condensations involved in the reaction of fluorene with dibiphenylene-ethylene.

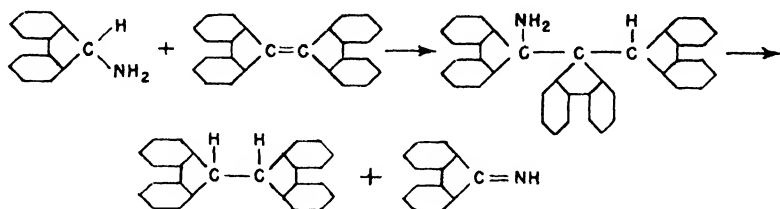
**TYPE I.** A reaction involving a condensation of fluorene with dibiphenylene-ethylene yields a stable end-product, namely, tribiphenylene-propane.



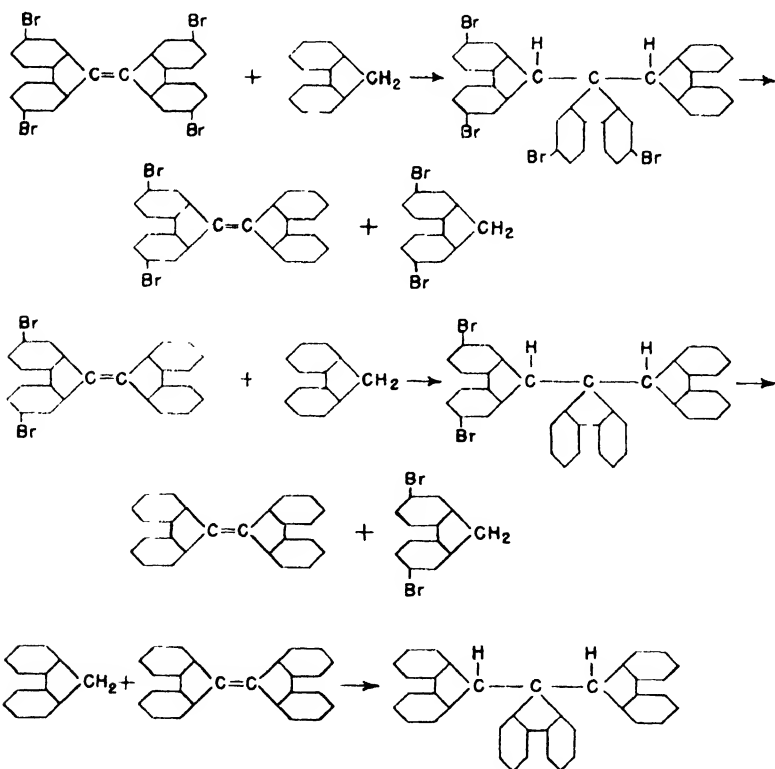
\* "Potentially carcinogenic" is an expression used in designating carcinogenesis with reservation. For example, if the compound is toxic, the animal will die before carcinogenesis sets in. Other considerations are (1) the limiting range of solubility of the carcinogen in the cell tissue, and (2) type and strain of animal used.



TYPE II. A reaction of dibiphenylenc-ethylene with a 9-substituted fluorene, in which the substituent has a potentially labile hydrogen, yields an unstable intermediate which undergoes intramolecular oxidation-reduction.



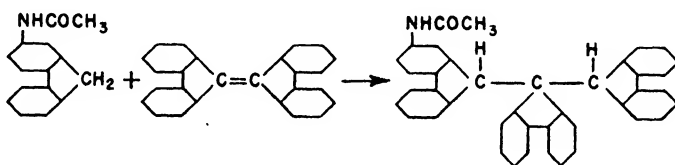
TYPE III. The most interesting condensation, which is the basis of the hypothesis, involves a reaction of 2,7,2',7'-tetrabromodibiphenylene-ethylene with fluorene yielding tribiphenylene-propane and 2,7-dibromofluorene. The complete reaction involves three condensations and two dismutations, as follows:



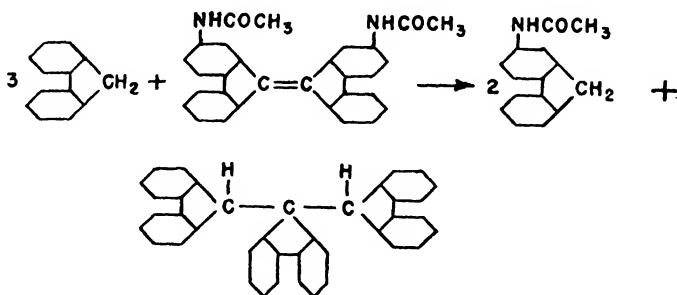
These three types of Michael condensations definitely demonstrate the great activity of the methylene group in the fluorene molecule and of the ethylenic carbons in dibiphenylene-ethylene, as well as the influence exerted by certain substituents in certain positions of the fluorene molecule and of the fluorenylidene nuclei of the ethylene.

**2-Acetylaminofluorene.** Now let us consider the differences between fluorene and 2-acetylaminofluorene. The most significant difference between these two compounds is that 2-acetylaminofluorene is carcinogenic<sup>1, 3, 35, 38</sup> whereas fluorene is not.<sup>5, 14-17, 24, 32</sup> Chemically, the difference between these two compounds is that the hydrogens on the 9-carbon of 2-acetylaminofluorene are more active than the corresponding hydrogens of the parent hydrocarbon.<sup>29</sup> On the basis of related work,<sup>27, 28</sup> the two following predictions are made.

(1) 2-Acetylaminofluorene, like fluorene and 2,7-dibromofluorene, will condense with dibiphenylene-ethylene in a manner corresponding to a TYPE I condensation.

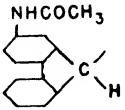
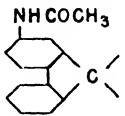


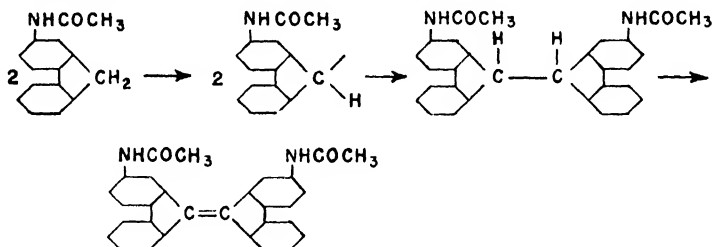
(2) Fluorene will condense with 2,2'-di(acetylamino)dibiphenylene-ethylene in a manner corresponding to a TYPE III condensation, which is indicated by a composite equation of the five reactions involved.



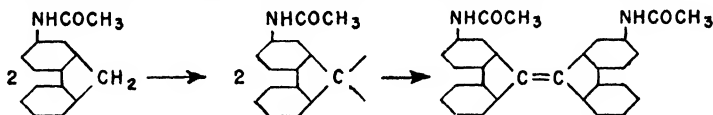
These two predictions, therefore, show that the chemical difference lies not in the fluorenes but in their dehydrogenated dimers, namely, the ethylenes. It is, therefore, deduced that 2-acetylaminofluorene, as such, is not carcinogenic but that the carcinogen is 2,2'-di(acetylamino)dibiphenylene-ethylene, because this compound is chemically different from dibiphenylene-ethylene, the latter being non-carcinogenic.<sup>2</sup> Hence, it is assumed that 2-acetylaminofluorene undergoes enzymatic dehydrogena-

tion forming 2,2'-di(acetylamino)dibiphenylene-ethylene. Whether the

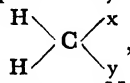
dehydrogenation proceeds yielding a fluorenyl group  or a fluorenylidene nucleus  is an open question. If the fluorenyl group is formed, then dehydrogenation would involve three steps for the ethylene formation.



On the other hand, if bivalent carbon is formed, we would have



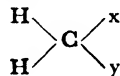
The course of this reaction could be obtained indirectly, namely, by determining whether 2,2'-di(acetylamino)dibiphenylene-ethane is carcinogenic or not. If the ethane is carcinogenic, then it should be capable of being dehydrogenated to the ethylene. However, if it proves to be a non-carcinogen, then the dehydrogenation would involve the other mechanism and, furthermore, it may suggest a chemotherapeutic attack on the problem of carcinogenesis.

The material which is transformed into cancerous tissue is designated as cell substance, and it is assumed that it has two potentially labile hydrogens. For convenience, the chemical formula, , is assigned

to it where  $x$  and  $y$  are moieties of the cell substance. Naturally, the moieties of the cell substance in the different parts of the animal, such as liver, intestines, lungs, etc., would be different from one another. Furthermore, different strains of the same animal, different animals, age, sex, and other factors might possibly affect certain variations in the moieties. That cell substance which has the most labile hydrogens may under suitable conditions turn into cancerous tissue.

On the basis of the above assumptions, we now have a chain of six reactions, the first being a composite reaction involving enzymatic dehy-

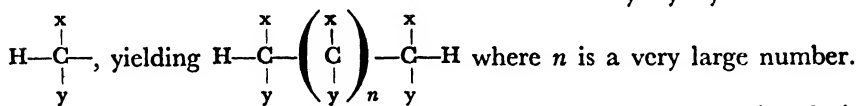
drogenation of 2-acetylaminofluorene yielding 2,2'-di(acetylamino)-dibiphenylene-ethylene, and the next five reactions involving a TYPE III condensation of the above ethylene with the cell substance,



yielding a dehydrogenated trimer of cell substance, *e.g.*,  $\text{H}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\text{H}$  and the original 2-acetylaminofluorene, the latter repeating the chain of reactions with fresh cell substance.

The condensation product of cell substance  $\text{H}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\text{H}$  has two potentially labile hydrogens and can grow in magnitude by enzymatic

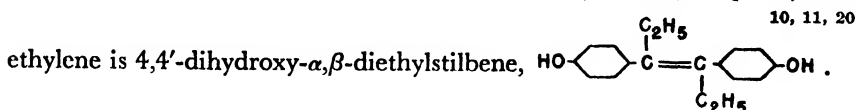
dehydrogenation and condensing either with  $\text{H}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-\overset{\text{x}}{\underset{\text{y}}{\text{C}}}-$  or with



Regardless whether  $x$  and  $y$  remain  $x$  and  $y$  or metabolize to  $x'$  and  $y'$ , the unlimited and uncontrolled growth of cancer tissue may involve the building-up of a very long carbon chain of cell substances involving a biochemical mechanism of chain reactions as shown in CHART 1.

Furthermore, according to this hypothesis, 2-acetylaminofluorene is not the only fluorene compound which is a precursor of a carcinogen. It is possible that 2,7-di(acetylamino)fluorene may possibly be even more carcinogenic than the *mono*-substitution product, because the presence of a substituent in the 7 position will block an oxidation reaction which also takes place *in vivo*, forming 2-acetylamino-7-hydroxyfluorene which is eliminated in the urine.<sup>4</sup> Other substituents in the 2 and 7 positions of the fluorene molecule, having electronegativities within certain limits in the range of that of the acetyl amino group, may also make those fluorene derivatives carcinogenic. From a knowledge of either the relative electronegativities of the substituents or the chemical activity of the fluorene derivatives, it may be possible to predict which compounds will be carcinogenic and which will not.

**4,4'-Dihydroxy- $\alpha,\beta$ -diethylstilbene.** A carcinogenic compound having an ethylenic structure similar to 2,2'-di(acetylamino) dibiphenylene-



Its parent compound, stilbene, like dibiphenylene-ethylene, is noncarcinogenic.<sup>23</sup> In fact, stilbene is chemically so unreactive that it does not

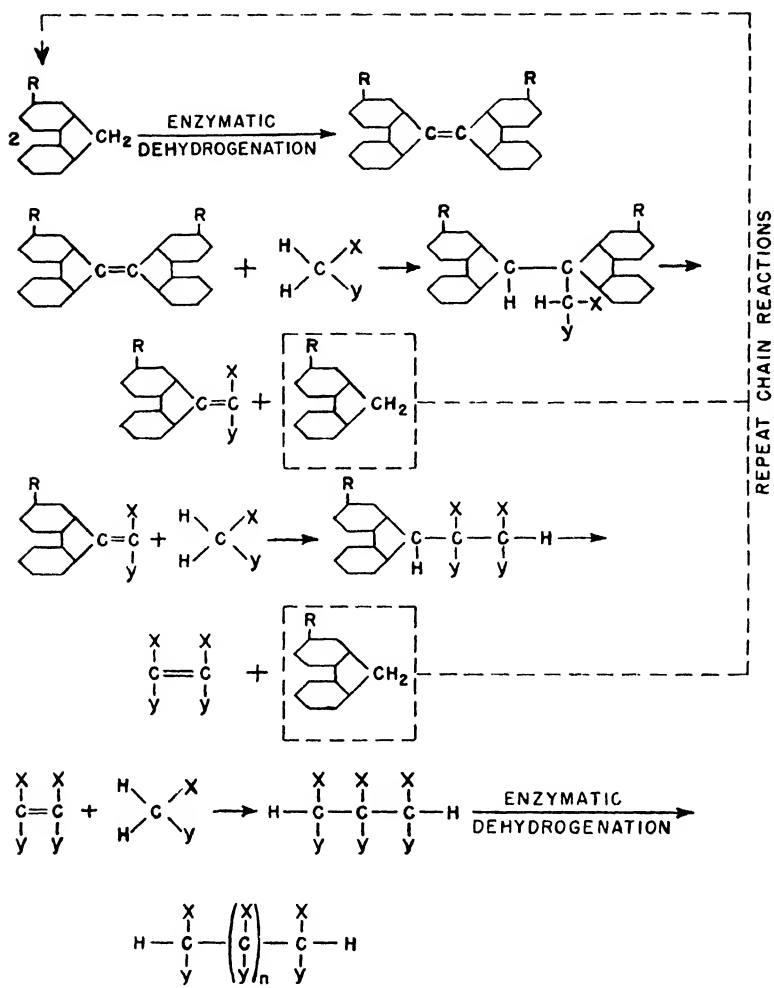
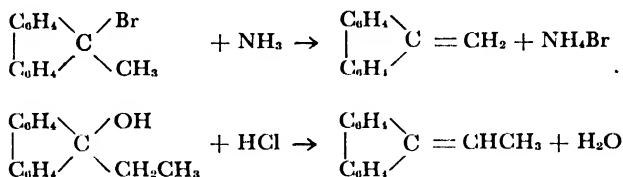


CHART 1. A possible mechanism of the genesis of cancer initiated by 2-acetylaminofluorene. End product of chain reactions is underscored. R = -NHCOCH<sub>3</sub>; x and y = moieties of cell substance; n = a very large number.

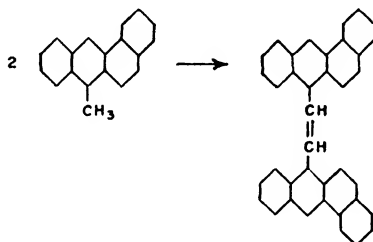
react with the very active 9-aminofluorene<sup>25</sup> (TYPE II condensation). However, its ethylenic carbons appear to be activated by the presence of certain substituents in certain positions of the molecule as indicated in its structure. The biochemical mechanism of carcinogenesis resulting from 4,4'-dihydroxy- $\alpha,\beta$ -diethylstilbene follows the same pattern as that involving 2-acetylaminofluorene. However, I would not expect 4-propylphenol, which is supposedly formed in the TYPE III condensation, to undergo enzymatic dehydrogenation and then condense to form the dihydroxy-diethylstilbene, for the hydrogens on the  $\alpha$ -carbon of a propyl group are not at all active.

**10-Methyl-1,2-benzanthracene.** A methyl group becomes activated when it is attached to the 9-carbon in fluorene.<sup>20</sup> When an ethyl group is attached to the 9-carbon in fluorene, the methylene is activated.<sup>8</sup> These activities are illustrated by the following reactions:

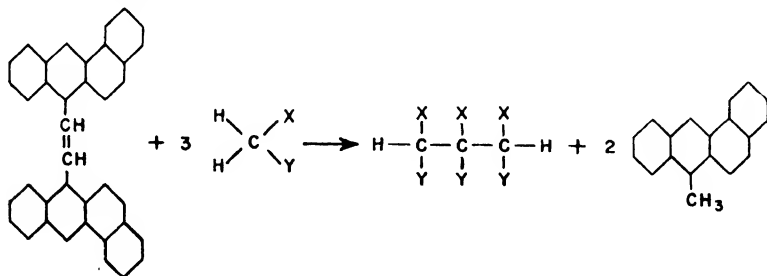


However, a propyl group or a longer chain have not been reported to possess such activation.

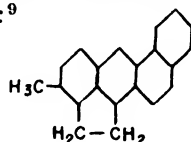
In 1,2-benzanthracene, the 10-carbon is the active center of the molecule,<sup>9</sup> and when either a methyl or an ethyl group is attached to the 10-carbon, each one in turn becomes activated, whereas a propyl group remains inactive. These methyl and ethyl benzanthraces have been found to be carcinogenic.<sup>33</sup> Additional substituents enhance the carcinogenic activities when present in certain positions of the molecules, and when these same substituents are located in other positions, the activities are either decreased or completely nullified.<sup>33</sup> This, of course, is expected because of the alteration in the electronic configuration of the molecule. According to the proposed hypothesis, 10-methyl-1,2-benzanthracene is a precursor of a carcinogen and undergoes enzymatic dehydrogenation forming the carcinogen, *di*-1,2,1',2'-benzanthraces-10,10'-ethylene.



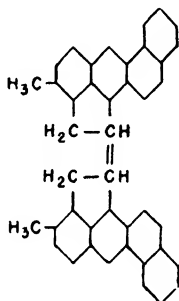
The formation of the dehydrogenated trimer of cell substance is indicated by the following composite equation.



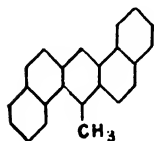
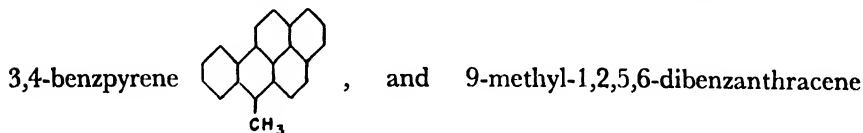
**20-Methylcholanthrene.** In the genesis of cancer, the study of 20-methylcholanthrene occupies a preeminent place. Hartwell in his survey<sup>12</sup> reported 86 references for this compound. The active group in methylcholanthrene is the methylene in the 15 position:<sup>9</sup>



The influence of the methyl group in the 20 position, if any, is to enhance the reactivity of the already activated 15-carbon, for cholanthrene is also a very powerful carcinogen. This compound, according to the hypothesis, follows the same pattern of biochemical chain reactions, the first step being the formation of a dehydrogenated dimer.

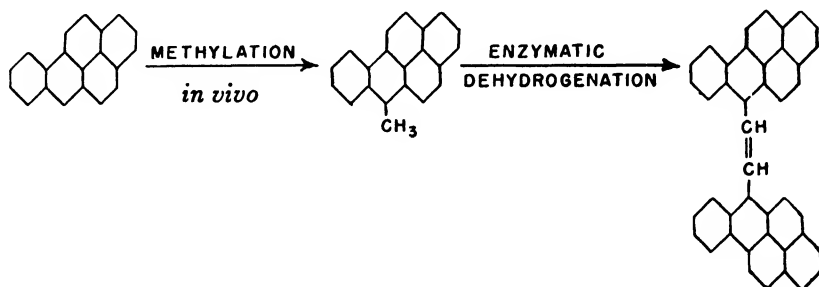


**3,4-Benzpyrene and 1,2,5,6-Dibenzanthracene.** Such powerful carcinogens as 3,4-benzpyrene and 1,2,5,6-dibenzanthracene which do not have a methyl group, it is assumed, obtain it in a metabolic process in the animal tissue. This assumption is not so far fetched when we consider the following facts: (1) Miller, Miller, and Baumann<sup>22</sup> found that, when *p*-methylaminoazobenzene was fed to rats, the liver contained both *p*-aminoazobenzene and *p*-dimethylaminoazobenzene in addition to the dye fed. This shows that the rat is capable of methylating an organic compound. (2) du Vigneaud and associates,<sup>36, 37</sup> also working with rats, definitely showed betaine, choline, and sarcosine to be effective methyl donors. (3) The methyl derivatives, namely, 5-methyl-



, which are assumed to be intermediates, were reported

by Shear and co-workers<sup>33</sup> as being even more powerful carcinogens than the unmethylated hydrocarbons. Using benzpyrene as an example, the preliminary steps in this mechanism of reactions are as follows:



The rest of the reactions are, of course, analogous to the examples previously cited.

**Azo Compounds and Amines.** In all the compounds thus far considered, the carcinogenic activity was focused on the ethylenic linkage. Since a common chemical relationship was shown to exist between dibiphenylene-ethylene and azobenzene,<sup>25, 28</sup> it was not at all surprising to find that certain azo compounds are carcinogenic. Hartwell<sup>12</sup> reported about a dozen azo compounds to be carcinogenic out of 39 which were examined through 1939. The azo compound that was studied most is

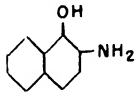
*o*-aminoazotoluene Cc1ccccc1N=Nc2ccccc2N (Hartwell cited 68 references)

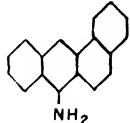
and next in line is *p*-dimethylaminoazobenzene CN(C)c1ccc(N=Nc2ccccc2)cc1 with 35 references. Both of these compounds are carcinogenic, whereas azobenzene is not.<sup>18, 19, 21</sup>

In carcinogenic studies of azo compounds, we do find some experimental evidence which is in harmony with the hypothesis. For example, Miller and Miller<sup>22a</sup> recently found a "tight combination, presumably chemical" between an aminoazo dye and a cellular constituent in the liver of rats. The dye could not be extracted from the isolated crude liver protein by boiling organic solvents, but it could be extracted after the protein had been degraded with hot alkali or trypsin. This seems to indicate that the combination is chemical and that the most probable reaction would involve the saturation of the azo linkage. Another significant point reported in the same paper, which conforms to the hypothesis, is that the bound dye did not occur in the tumors which were formed in the liver. In another important paper on azo dyes, Stevenson, Dobriner, and Rhoads<sup>34</sup> reported that reduction takes place at the azo linkage, yielding reduced split products which conform to the mechanism postulated in this paper (see CHART 2).



Dimethyl-*p*-phenylenediamine<sup>18, 19</sup> and *p*-phenylenediamine<sup>18, 21, 30</sup> are not carcinogenic. Hence, it is not expected that these amines would undergo enzymatic dehydrogenation forming azo compounds. On the

other hand, such amines as 2-amino-1-naphthol <sup>13</sup> β-naph-

thylamine,<sup>13</sup> and 10-amino-1,2-benzanthracene <sup>33</sup> have been

reported to be carcinogenic. According to the hypothesis, these amines are capable of being dehydrogenated and then condensed to the azo compounds.

In general, the mechanism of reactions of carcinogenesis involving azo compounds follows the same pattern as 2-acetylaminofluorene.

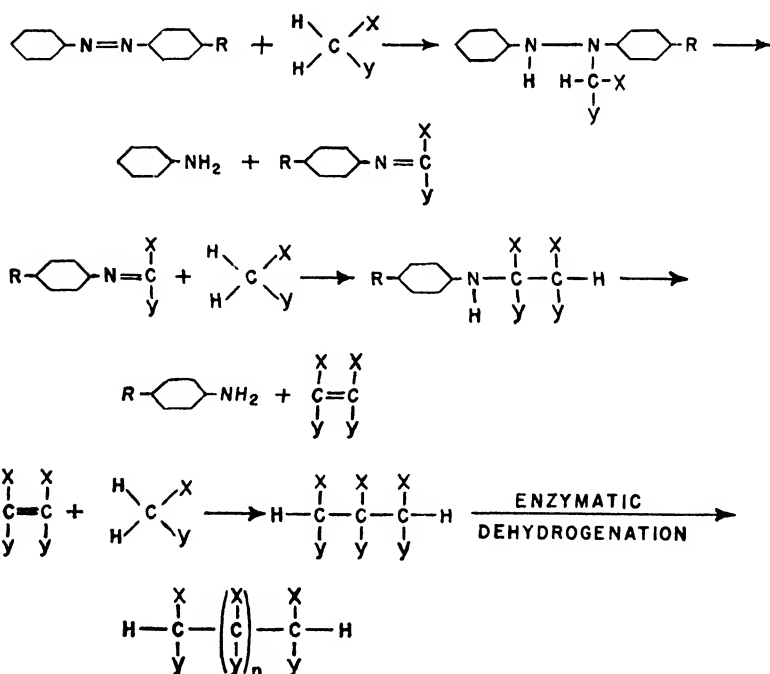
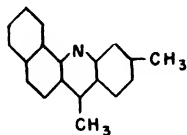


CHART 2. A possible mechanism of the genesis of cancer initiated by an azo compound. R = --N<sub>2</sub>(CH<sub>3</sub>)<sub>2</sub>; x and y = moieties of cell substance; n = a very large number.

**Discussion.** In this communication, the following seven different types of aromatic carcinogenic compounds have been considered: (1) a fluorene, (2) a stilbene, (3) an aromatic compound containing a methyl group, (4) an aromatic compound containing a 5-membered

ring, (5) a cyclic aromatic hydrocarbon, (6) an azo compound, and (7) certain amines. Most of the aromatic compounds thus far reported to be carcinogenic may fall in line with some one of the types listed. According to the hypothesis, certain organic compounds are carcinogenic by virtue of having or being capable of forming *in vivo* a very reactive double bond. The electronic configuration of these molecules is such that these active compounds react with the cell substance in a manner corresponding to TYPE III Michael condensation. In this paper, the active double bond has been suggested to exist between two carbons and between two nitrogens. However, future work on carcinogenesis may possibly reveal other such active linkages. Some indirect evidence favoring the ethylene formation is suggested in Ruffilli's experiments with carcinogenic hydrocarbons.<sup>31</sup> The author implanted pellets of paraffin containing methylcholanthrene or benzantrhene under the skin of animals for three months and then upon removal and careful purification isolated in each case a yellow colored product. The yellow color of the resulting substances suggests that it may be due to ethylene formation, for it is well known that conjugation of double bonds frequently obtain colored compounds.

The difference between carcinogenic and non-carcinogenic compounds seems to be that the carcinogens undergo a TYPE III condensation, whereas the other compounds may undergo a TYPE I or TYPE II or may be incapable of undergoing any condensation. For example, dibiphenylene-ethylene will undergo a TYPE I and a TYPE II, whereas stilbene will not undergo any condensation. A compound such as 10-cyano-1,2-benzanthracene is not carcinogenic because the cyano group blocks it from undergoing the necessary dehydrogenation and ethylene formation. Shear and co-workers<sup>33</sup> reported that the crystalline compound was seen at the injection site in all of the survivors which were killed in the sixteenth month. Two types of benzacridines recently reported by Buu-Hoi<sup>6</sup> fit into this hypothesis very nicely. 5,8-Dimethyl-1,2-benzacridine,



is carcinogenic, for the position of the cyclic nitrogen

does not affect any of the anticipated reactions of the methyl group attached to the active *meso* carbon. On the other hand, the isomeric 5,7-

and 5,8-dimethyl-3,4-benzacridines

are not active, for the active center of these molecules is where the nitrogen is located and structurally the nitrogen is fully saturated. Cook and Preston<sup>7</sup> reported that, "1,2,5,6-Dibenzfluorene has feeble cancer-producing activity and has also a very pronounced effect in inhibiting the growth

of tumors." This statement sounds paradoxical but seems to be plausible in light of the hypothesis.

A point worth considering is that, more often than otherwise, it is extremely difficult to obtain reactions *in vitro* that occur *in vivo*. In the case of the fluorene compounds and possibly the azo compounds, it is rather fortuitous that the proper reactants and a favorable environment to make such reactions *in vitro* possible were obtained, which may possibly occur with cell substance in an analogous manner *in vivo*. Should the other carcinogenic compounds not respond to the same reactants and environment, then it may be necessary to change either one or both or possibly modify the chemical architecture of the carcinogenic hydrocarbons.

Another method of establishing the validity of the hypothesis lies in a carcinogenic study of a series of new compounds which heretofore have not been tested. According to the hypothesis, the following compounds may be potentially carcinogenic: 2,2'-*di*(acetylaminio)dibiphenylene-ethylene (I), 2,7,2',7'-*tetra*(acetylaminio)dibiphenylene-ethylene (II), other dibiphenylene-ethylene derivatives capable of undergoing a TYPE III condensation, 10,10'-azo-1,2,1',2'-benzanthracene (III), *di*(1,2,1',2'-benzanthracene)-10,10'-ethylene (IV), *di*(1,2,5,6,1',2',5',6'-dibenzanthracene)-9,9'-ethylene (V), *di*(3,4,3',4'-benzpyrene)-5,5'-ethylene (VI), and dehydrogenated dimers of 20-methylcholanthrene and cholanthrene (VII and VIII)—see CHART 3.

These are but a few of the compounds which may be potentially carcinogenic. A very extensive list could be prepared by considering the dehydrogenated dimers of many of the known carcinogenic hydrocarbons.

With this as a working hypothesis, the following lines of work are suggested: (1) Condensation reactions relating to 2-acetylaminofluorene. (2) Syntheses and reactions of 2- and 2,7-substituted fluorenes with substituents of graded electronegativities. (3) Condensation reactions of azo compounds. (4) Syntheses and reactions of ethylenes derived from various types of carcinogenic aromatic compounds. (5) Biological tests for carcinogenesis of the suggested new compounds, principally the ethylenes and some ethanes. (6) Biological tests of other compounds obtained in the chemical studies mentioned above.

The importance of this hypothesis is that it may not only shed light on the genesis of cancer but may also suggest a scientific method of approach in the study of chemotherapy. As yet, there is no clear-cut method in the study of this extremely important problem.

## SUMMARY

A hypothesis of the genesis of cancer based on a correlation of chemical and carcinogenic activities of 2-acetylaminofluorene has been formulated showing that 2-acetylaminofluorene, as such, is not carcinogenic but that it may be a precursor of the carcinogen, 2,2'-*di*(acetylaminio)dibiphenyl-

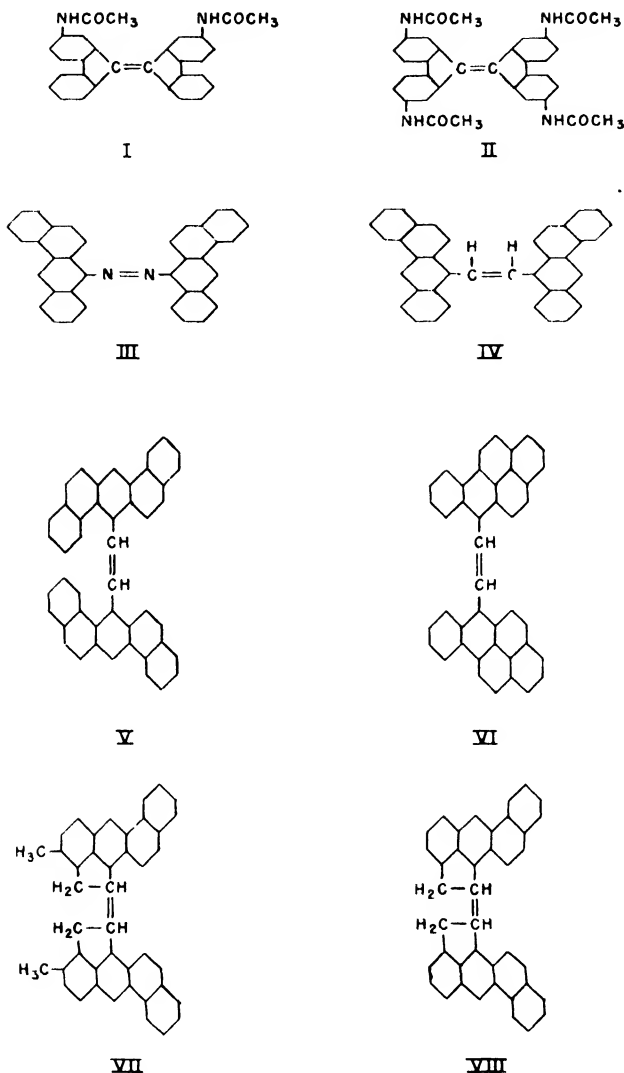


CHART 3. New compounds which may be carcinogenic.

ene-ethylene, which is obtained by enzymatic dehydrogenation. A chain of reactions has been formulated showing a chemical picture of the genesis of cancer involving the regeneration of the carcinogenic compound which repeats the chain of reactions on fresh cell substance. It has also been postulated that other fluorene compounds having certain substituents in the 2 and 2,7 positions may be precursors of carcinogenic compounds.

Other carcinogenic representatives of the following classes of compounds have been considered: stilbenes, aromatic compounds containing

a methyl group, a five-membered ring and cyclic hydrocarbons, azo compounds, and amines. It is postulated that the reactive portion of the carcinogenic molecule is either the ethylenic or azo groups. Those compounds which do not have these configurations obtain one or the other by enzymatic dehydrogenation.

It has also been postulated that the difference between cancer tissue and normal tissue is that the former consists of a very long carbon chain of cell substance.

On the basis of the proposed hypothesis, some hitherto unprepared organic compounds are predicted to be carcinogenic.

New lines of chemical and biological studies relating to carcinogenesis are suggested.

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## THE CHEMOTHERAPY OF FILARIASIS\*

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# FILARIASIS

By L. T. COGGESHALL

*Department of Medicine, The University of Chicago, Chicago, Illinois*

Filariasis, an infection of man and lower animals, has been known to exist since the earliest records of history. Yet, despite the fact that there are probably 1,800,000 to 2,000,000 infected individuals scattered over the globe and it once was prevalent in the southeastern United States, the disease held little interest in this country until World War II. At that time, it became apparent that several thousand United States servicemen had contracted the infection on certain Pacific isles. It became a subject of considerable concern, and great effort was expended in a search for effective chemotherapeutic agents to combat the infection. The abstracts for the present conference indicate that considerable achievement has crowned their efforts. As a background to a symposium on chemotherapy, the following remarks will be devoted to the general aspects of the disease.

The filarial organisms belong to the group of nematode parasites and there are six closely related species—*Wuchereria bancrofti*, *W. malayi*, *Onchocerca volvulus*, *Mansonella ozzardi*, *Acanthocheilonema perstans*, and *Loa loa*—in addition to the filarial-like organism *Dracunculus medinensis*, which produce infections in man. In lower animals, filariasis occurs in horses, cattle, goats, swine, dogs, birds, frogs, and many other lower forms of vertebrate life.

## HISTORY OF *WUCHERERIA BANCROFTI*

Due to elephantiasis, which produces grotesque deformities and which is the chief serious complication of filariasis, the disease has been recognized for centuries, but little was known about its cause until 1863, when Demarquay found the embryonic forms, microfilariae, in hydrocele fluid. In 1866, Wucherer found microfilariae in chylous urine, and Lewis found the same forms in the circulating blood in 1872. The adult worm was first noted by Bancroft in 1876.

The mosquito was incriminated as a vector in 1878 by Manson, who also noted at the same time that the microfilariae appeared in large numbers at night but were absent from the human blood during the day.

The demonstration that the mosquito was an intermediate host was soon followed by a complete understanding of the life history of *Wuchereria bancrofti*. The susceptible mosquito ingests microfilariae in a blood meal and, within a few minutes, they lose their protective sheath and make their way to the body cavity and thoracic muscles of the mosquito. Here, they undergo further development, but do not become sexually mature although they move in pairs. When the mosquito bites a normal indi-

vidual, the larvae escape and enter the puncture wound or penetrate unbroken skin. From here, they soon find their way into the peripheral lymphatics and eventually migrate to the larger lymphatic trunks. Sexual maturity is attained and mating occurs in the lymph nodes. The female rapidly develops a huge uterus filled with embryonic forms which are then discharged into the bloodstream via the lymphatic trunks. This completes the cycle, and it is impossible for the microfilariae to attain maturity without passage through an appropriate mosquito and reintroduction into man.

**History of Filariasis in the United States.** With the advent of slavery, *W. bancrofti* filariasis was transported to this country and succeeded in maintaining a foothold in the southeastern states, particularly in the region of Charleston, South Carolina. It was also found as far north and west as Indiana. However, when slave trade was no longer carried on, the incidence of the disease gradually decreased until about 1925. Since then, little or no evidence of filariasis has been reported. Actually, on a climatic basis, *W. bancrofti* requires a mean temperature of about 80° F. and an average humidity of 60 per cent or more. Since there are very few areas in the United States in which these climatic conditions prevail, it is not surprising that the disease disappeared after its source had been severed.

The next episode was encountered when United States troops were transported to the western Pacific islands where filariasis is especially prevalent. Here, military necessity forced the staging areas to be closely associated with native compounds, and within a few months numerous cases of filariasis appeared. The early manifestations of filariasis have received little attention, because most of those interested in the infection have devoted their studies to the more advanced stages of the disease. Actually, many believed that the white man was not susceptible, although conclusive evidence to the contrary was present in many reports.

In the servicemen, the first symptoms were lymphangitis, lymphedema, and lymphadenitis. In over 15,000 men infected, these symptoms appeared in only 0.6 per cent during the first month after exposure. Nine per cent had an incubation period of over 18 months, while the greatest number noted their first symptoms during the tenth month. As this infection is largely one of the lymphatic system, the symptoms were confined primarily to the lymph nodes in the axillae, groins, and spermatic cords. The lymphangitis occurred along the course of the major lymphatic vessels in the arms and legs, always appearing proximally and extending distally in contrast to the behavior of lymphangitis of bacterial origin. There was an associated fever of usually 101° to 102° F., some malaise, and a mild leucocytosis. These findings of the acute infection lasted, as a rule, about 4 to 7 days and there were then considerable periods of freedom from incapacitating abnormalities, usually for two months, after which they would reappear. Strenuous activity would frequently incite an exacerbation of symptoms.

The diagnosis of filariasis in the servicemen was frequently questionable because it was dependent upon history of exposure and the above-mentioned symptoms. However, biopsies of large lymph nodes were made in a sufficient number of instances in which living or degenerating adult worms were demonstrated, so that the evidence was more than presumptive. Although many of the men were observed closely for as long as four years after their initial symptoms, it was not possible, in the author's experience, to demonstrate successfully the presence of microfilariae in the bloodstream. This was not surprising, because the greatest total exposure period was less than two years in any man and this period would not account for a sufficient number of adult worms which could, in turn, liberate enough microfilariae to make them microscopically detectable. Even in the natives, where infection may occur daily for many years, it is unusual to find more than 60 per cent of the people in any area having circulating microfilariae. As was anticipated, the infections in the servicemen gradually but definitely died out without treatment, because the defense mechanisms in the body were sufficient to control these light infections.

During the period of the acute symptoms and early convalescence, the chief difficulty was a psychological one, which it was possible to eliminate, in most instances, by careful explanations of the disease process and reassurance that the end-result would be a favorable one. Actually, less than 5 per cent of the total number infected are receiving compensation for disability due to this infection at the present time.

The question as to whether filariasis imported into this country from the Pacific area would constitute a public health menace can be answered by the finding that in no instance were microfilariae detected beyond reasonable doubt and, in the few cases which reportedly showed an occasional organism, the number was far below that required to initiate infection in mosquitoes.

**Symptomatology and Pathology of Chronic Infections.** Filarial infections, in common with other tropical parasitic infections, have as their chief characteristic a tendency to run a chronic course. Microfilariae transfused in large numbers from infected donors are known to exist for as long as two years without undergoing further development in the human host. Likewise, in natural infections, in individuals removed from endemic areas and in whom microfilariae are demonstrable, it may be noted that these embryonic forms persist for many months or even years. As would be expected in a long-term chronic infection, the symptomatology is varied. Initial stages are associated with the symptoms described in the servicemen, namely, inflammation and dilation of the lymphatic channels, hypertrophy and inflammation of the lymphatic glands, with localized areas of lymphedema. As the patients are constantly being reinfected, there is continued injury to the tissues of the lymphatic system which, in many instances, produces a blockage

of that system by fibrosis with an end result of elephantiasis. Elephantiasis is to be regarded as a complication occurring, at most, in not more than 7 per cent of chronic infections, and it usually appears in the lower extremities, the genitalia, and in the mammary glands of the female. Asymptomatic filariasis is very common in all parts of the world and, in certain areas, accounts for the major proportion of those infected.

Frequent controversy has arisen as to whether the indurated tissue associated with elephantiasis is the result of secondary bacterial invasion by streptococci. Although a plausible theory, this has never been substantiated and at the present time is generally held in doubt. Certainly, the lymphangitis is not typical of that seen in streptococcal infections not associated with filariasis, and, likewise, it does not respond to the chemotherapeutic or antibiotic agents which are known to have a specific effect on the streptococcus.

**Immunology.** Like many of the other parasitic infections, a high degree of immunity is not present in filariasis since individuals are susceptible to reinfection. As a matter of fact, it is common practice for infected persons to leave an area when filarial manifestations become troublesome, and in a filarial-free area all symptoms of a temporary nature subside. Yet, upon return, the individual seems just as susceptible as he was at the initial exposure. Although there is great need for further investigation into the immunological aspects of filariasis, there is no evidence, at present, which would indicate that sufficient resistance could be obtained from a vaccine which would insure protection for one who contemplates living in endemic areas.

**Prophylaxis and Control.** At present, there is no chemotherapeutic agent which will prevent an individual from becoming infected, and the major effort has been directed towards the control of the vectors which transmit filariasis. In general, the mosquito vectors for this disease have been found easier to eliminate than those associated with some of the other tropical infections. The requirements for successful transmission seem to be more exacting, which permits more effective interference when control measures are instituted. Likewise, many of the vectors live in very close contact with the native populations and only those vectors in most immediate contact show high rates of infection. For example, in Samoa, it was discovered that the principal vectors were not infected when captured as short a distance as 150 yards from the center of the native villages, while as many as 40 per cent of those captured in the center of the village were infected. Actually, a difference in the rates of infection could be detected in mosquitoes captured on the windward side of the village as contrasted to those captured on the leeward. Thus it can be seen that minimal control efforts in the immediate neighborhood of any village would successfully eliminate the chief source of difficulty

## OTHER FILARIAL INFECTIONS OF MAN

**Loiasis.** Loiasis, or *Loa loa* infection, is caused by the worm of that name. This parasite migrates in the subcutaneous tissues and gives rise to localized swelling known as Calabar swellings, and it frequently produces a local reaction in the conjunctiva. It is transmitted by tabanid flies (*Chrysops*), and has a very widespread distribution in western and central Africa, particularly in the larger river basins. The symptoms of this infection are restricted largely to the swellings which occur on the course of the path of the migrating worm. They are painful, temporary in nature, and when punctured usually reveal microfilariae. They probably represent an allergic phenomenon. Microfilariae can also be detected in the peripheral bloodstream. Prevention is restricted entirely to the control of the vector and there is no specific treatment.

**Onchocerciasis.** This disease is produced by the parasite *Onchocerca volvulus*, discovered in 1893, and, like loiasis, is characterized by numerous widespread subcutaneous nodules over the body. It is transmitted by the gnat, *Simulium*. It is distributed in tropical Africa over the wide central belt and is also prevalent in the Central American countries, particularly Mexico and Guatemala. In the latter areas, it may be found in the coffee plantations at elevations as high as 6,000 feet. The microfilariae of this worm are found in the subcutaneous tissue, particularly in the subcutaneous nodules, but are not encountered in the bloodstream. The *Simulium* fly develops in swift-running streams and infected flies are usually not detected at great distances from this source. The tumors vary in size from a pea to a hen's egg, are extremely painful, but subside without rupture. They are encountered at all ages and have been noted in children as young as two months of age. The distribution of the nodules varies in different localities. For example, in Africa, it is rare to find them on the head, while in Central America this is the common site. Like *Loa loa*, this parasite also exhibits a specific localization in the eye where it frequently penetrates the eyeball and has been known to invade the optic nerve with resulting blindness. Diagnosis is confirmed by subcutaneous biopsies or demonstration of microfilariae from the nodules. Specific treatment has not been particularly effective.

**Dracontiasis.** This illness, also known as guinea-worm disease, is produced by the closely related filaria worm, *Dracunculus medinensis*, which frequently grows until it is three feet long and its presence is noted beneath the skin, particularly in the lower extremities. It is widely distributed in the tropics, occurring in Asia, the Middle East, Africa, and South America. It has not been reported from the Pacific area. Unlike the other filarial infections, it is transmitted by the water flea (cyclops) which becomes infected by the embryos of the adult worm escaping from an aperture in the skin of the infected individual.

This aperture coincides with the opening of the uterus of the adult female worm which is stimulated and expresses larvae when coming in contact with water while the human host is wading, washing, etc. When the infected cyclops are swallowed in drinking water, they are immediately killed by the gastric juice and the larvae escape, penetrating the intestinal wall and migrating to the surfaces of the body where they have completed their development into worms many centimeters long. The only treatment is surgical removal of the adult worms, or, more commonly, they are slowly wound onto a stick or thorn and gradually removed from the body over a period of several days. Prevention can only be accomplished by purifying, or removing the source of, contaminated water.

### SUMMARY

Filariasis is a disease in humans which affects many thousands of individuals in the tropical and subtropical areas of the world. There is no doubt that the economy and welfare of many lands are seriously affected by these infections in spite of the fact that they are associated with very low mortality rates. Until the present, there have been no chemotherapeutic agents which have demonstrated their ability to influence the infection in man.

The chief method of control must lie in the suppression or eradication of the vectors, a task made much easier since the discovery of more effective insecticides. In this respect, we cannot be hopeful of too much success, since all diseases of high morbidity and low mortality are usually associated with indifference on the part of the general public, and only those directly concerned with public health responsibilities can be expected to exert any considerable influence. All too frequently, their efforts are so restricted, in spite of general educational campaigns, that widespread benefits are not obtained.

As stated above, this discussion has not considered chemotherapy. However, since there is little prospect of a successful immunizing agent, and since the vectors and procedures for their control have been recognized for at least two decades without apparent diminution of the incidence of the disease, the introduction of an effective chemotherapeutic agent would be a most welcome adjunct to those charged with the responsibility of eliminating this infection.

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# GENERAL EXPERIMENTAL METHODS USED IN STUDYING FILARICIDES

By REDGINAL I. HEWITT

*Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.*

The extensive use of cotton rats (*Sigmodon hispidus*), naturally infected with filaria (*Litomosoides carinii*), during the past three years for the evaluation of drug activity, has largely supplanted all previous known *in vivo* methods in the search for new filaricides. This is particularly true in screening tests, since the relatively small size of this host allows the testing of small chemical samples. Dogs infected with *Dirofilaria immitis* provide a useful host-parasite relationship for confirmative or comparative assays, but their large size discourages their use in routine screening programs.

In addition to the advantages of small size and ready availability of the cotton rat, several investigators have provided useful information relative to the biology of untreated infections. Studies have been made on microfilarial periodicity,<sup>1</sup> transmission,<sup>2</sup> rate of growth and maturity of the worm,<sup>3</sup> developmental anatomy of the larvae and adult worms,<sup>4</sup> production of quantitative infections through the use of the vector,<sup>5</sup> and pathological changes in untreated infections.<sup>6</sup> Papers have also appeared relative to the effects of several different types of chemical compounds in naturally acquired cotton-rat filariasis. These include Neostam and Neostibosan,<sup>7</sup> Stibanose,<sup>8</sup> various mercury and antimony compounds,<sup>9</sup> Streptothricin, Urea stibamine, Vanadium gluconate, Acranil, Plasmochin, *p*-(-Phenylphenoxy)aniline, Phthalic acid, Penicillin, and Chloro-N- $\beta$ -dimethylphenethyl amine Hydrochloride,<sup>10</sup> cyanine dyes,<sup>11</sup> piperazine compounds,<sup>12</sup> and substituted phenyl arsenoxides.<sup>13</sup>

The accumulation of this information during a comparatively short period of time has provided a useful background for investigators interested in filaricides, and has advanced our knowledge regarding possible remedies for filariasis in human subjects.

The methods used for evaluating the effects of drugs in naturally acquired filaria infections in cotton rats are discussed in the present paper, together with the methods employed for *in vitro* studies and the bioassay of filaricides in *Dirofilaria*-infected dogs.

**Description of Naturally Acquired Infections with *Litomosoides carinii* in Cotton Rats.** *Litomosoides carinii* occurs as a natural parasite of the cotton rat in the southern United States, and in Central and South America. The adult worms are commonly found in the pleural cavity, but may occur occasionally in the peritoneal cavity as well. The female worms vary from 70 to 90 mm. and the male worms from 19 to



TABLE 1  
UNTREATED COTTON RATS—WEEKLY MICROFILARIA COUNTS

Rat No.	Microfilariae per 100 low power fields—weeks																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
150	75	88	176	420	220	540	420	332	220	212	1152	392	792	204	256	*		
151	34	22	56	160	232	460	216	400	920	552	596	536	668	288	480	648	1004	1070
153	56	168	276	332	398	700	332	948	*									
154	42	84	44	132	76	260	260	356	296	380	536	940	†	†	508	520	596	440
155	40	24	124	128	108	312	160	212	*									
156	116	88	300	396	264	580	176	604	*									
157	32	60	152	240	432	352	372	496	*									
158	40	148	26	68	40	372	268	308	*									
41	244	112	308	400	152	164	140	184	168	132	236	228	264	116	556	332		
108	252	360	56	142	104	564	112	152	312	200	76	92	176	248	140	312		
125	204	244	328	188	212	388	344	352	632	452	248	216	480	388	380	364		
182	200	204	120	304	344	400	652	364	816	500	528	652						
200	44	32	44	52	132	92	56	116	120	80	80							
193	56	48	24	52	68	†	44											
195	72	112	60	64	184	†	136	416	384	140	120	368	400					
198	212	120	92	160	472	†	600	284	632	236	592	520	1480					
201	20	22	18	8	0	†	*											
227	32	18	16	†	104	64	44	72	76	56								
241	68	16	84	†	208	100	144	200	164	168								
249	16	12	22	†	72	60	28	44	34	48								
362	40	56	68	14														
368	40	56	20	12														
388	560	410	240	720														
389	120	780	430	88														

† = no reading taken. \* = death.

TABLE 2  
UNTREATED COTTON RATS—MICROFILARIA COUNTS ON ALTERNATE DAYS

Rat No.		Microfilariae per 100 low power fields—days																	
		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35
41	244	156	240	112	316	675	248	308	268	180	336	128	396	152	368	164	164	196	
108	252	372	144	360	248	120	64	56	204	30	252	312	428	104	88	168	80	220	
125	204	336	212	244	576	244	192	328	268	124	296	22	308	212	260	224	272	452	
182	272	328	236	204	188	184	120	192	184	576	304	272	552	344	412	610			
200	34	56	64	32	34	30	44	24	24	44	52	60	96	56	7	46			
193	64	44	40	48	12	32	56	24	28	52	52	64	8	68	52	124			
195	116	60	108	112	108	104	64	60	68	196	64	44	140	184	72	244			
198	176	88	372	120	140	260	212	92	360	160	160	268	324	472	268	244			

TABLE 3  
UNTREATED COTTON RATS—MICROFILARIA COUNTS EVERY TWO HOURS

Rat No.	Microfilariae per 100 low power fields—hours																								
	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
227	32	14	30	18	6	10	16	12	14	14	30	14	44	44	20	60	30	56	88	76	56	60	40	108	44
241	68	32	36	4	44	40	104	96	88	44	18	48	28	56	128	100	84	172	184	144	180	152	116	76	96
247	60	24	16	96	18	10	56	52	42	8	32	40	28	16	76	54	40	56	60	76	26	*			
249	16	18	16	4	12	0	20	4	16	20	24	10	6	8	16	20	14	12	30	30	20	40	20	60	8

23 mm. in length.<sup>4</sup> Both sexes occur intertwined in masses of various sizes, and the total number present may exceed 500 worms.<sup>6</sup> It has been stated that, once the infection is acquired, the worms probably live throughout the life of the animal.<sup>6</sup> The visceral and parietal pleura may be covered with papillary nodules associated with the pathology of the infection.<sup>6</sup>

The vector of this parasite has been shown to be the tropical rat mite (*Liponyssus bacoti*),<sup>2</sup> and, in experimental infections, microfilariae appear in the peripheral blood as early as fifty days after transmission.<sup>3</sup> No periodicity is exhibited by the microfilariae,<sup>14</sup> although considerable fluctuation occurs in their numbers at different hours during the day, different days, or from week to week (TABLES 1, 2, and 3). In general, a steady increase occurs in the number of circulating microfilariae from week to week (TABLES 1 and 4).

TABLE 4

UNTREATED COTTON RATS.—PER CENT DIFFERENCE FROM ORIGINAL MICROFILARIA COUNT AT END OF OBSERVATION PERIOD

Rat No.	No. weeks observed	Per cent difference from original count	
		Increase	Decrease
150	14	254	
151	17	3047	
153	7	1593	
154	17	947	
155	7	430	
156	7	420	
157	7	1450	
158	7	670	
41	15	36	
108	15	23	
125	15	78	
182	11	246	
200	11	81	
193	6		22
195	12	455	
198	12	598	
201	4		100
227	9	75	
241	9	147	
249	9	200	
362	3		65
368	3		70
388	3	28	
389	3		27

The number of microfilariae present in the peripheral blood is not always correlated with the degree of infestation with adult worms, although, in general, rats showing extremely large numbers of microfilariae will exhibit large numbers of adult worms at autopsy.

Occasionally, untreated rats will show a small number of dead worms in the pleural cavity when autopsied.<sup>6, 12</sup> In 65 untreated rats autopsied

in these laboratories, 9 have shown a few dead worms, but massive deaths have never been encountered.

**Methods for Testing Drugs in Cotton Rats.** In common with all host-parasite relationships used for screening drugs experimentally, various methods are used by different investigators to obtain evaluations of filaricidal activity in the cotton rat. Manual treating, or the addition of the drug to the food, can be used successfully. The physical characteristics or known toxic effects of any given chemical compound frequently determine the route of administration, frequency of treatment, and length of treatment. In the case of new compounds, however, where no previous information regarding reactions in other animals is available, the choice of method for treatment and the duration of treatment are largely empirical.

In our program, provided nothing was known about a new drug made available for screening purposes, the degree of solubility in water determined the route of administration. Compounds soluble in water were given intraperitoneally, and insoluble compounds were administered orally in a 2.5 per cent starch suspension. If a sufficient amount of compound was available, the starting dose for oral administration was 200 mg. per kg., and for intraperitoneal administration 50 mg. per kg. If the animals died while under treatment, the dosage was halved and tried again. This was repeated until the test animals survived and the results were negative, or until positive effects were produced in a tolerated dosage. From one to three animals were used for each new compound, and treatment was continued twice daily for from two to four weeks. Autopsies were performed from one to four weeks after cessation of treatment. When positive filaricidal effects were observed, various dosage regimes were used in a large number of animals.

#### **Criteria Used for Evaluating Filaricidal Action in Cotton Rats.**

Since both adult worms and larvae of *Litomosoides carinii* occur in the cotton rat, as is the case with filaria infections in other animals, the evaluation of filaricidal action should take both stages of the worm into consideration. The destruction of microfilariae, without death of the adult worms, might have potential value in human infections for control and prophylaxis, although the ultimate goal for the therapy of filariasis in man would certainly seem to be the killing of adult worms.

Very few drugs have been shown markedly to reduce the number of microfilariae *per se* in the peripheral blood of cotton rats. Plasmochin,<sup>10</sup> some arsenicals,<sup>13</sup> and several piperazine compounds<sup>12</sup> will produce this effect. Following prolonged periods after treatment with Neostam, Neostibosan or Stibanose,<sup>7, 8</sup> the microfilariae gradually disappear in the cotton rat, but this is probably due to the death of the adult worms and is not brought about by a direct action upon the microfilariae.

Because of the failure of several filaricidal drugs to produce any measurable effect upon microfilariae *per se*, even though the majority

of adult worms were killed in cotton rats, all investigators have not used microfilaria counts as criteria for the immediate effectiveness of therapy in these animals. The correlation obtained by the present authors, however, between the effects of piperazine compounds on microfilariae and macrofilariae in cotton rats demonstrate that records of the microfilaria count are useful adjuncts to the total evaluation of filaricidal action.<sup>12</sup>

**MICROFILARIA COUNTS.** The estimation of the number of circulating microfilariae in cotton rats can be made from blood obtained from the cut tail, toes or ears. Culbertson and Rose<sup>7</sup> counted the number seen in 100 microscopic fields (x430) of fresh tail blood under a cover slip. Brown and Williams<sup>14</sup> devised a quantitative method by marking off a 0.9 cm. square on a slide and spreading the blood onto the marked area with a fine wire. The dried square was then stained with Giemsa's stain and every fifth row was counted under a high-power objective.

The present author prepared blood smears from the cut tails of rats, and these were stained with Giemsa's stain with or without dehemoglobinization. Generally, from 25 to 100 microscopic fields were counted under a low-power objective, but where very few microfilariae were present 100 fields were always counted. The count from all animals was then expressed per 100 microscopic fields. Counts were made on the day before treatment was initiated, and at various intervals thereafter, until an autopsy was performed.

Although all of the methods described are subject to error, it is not difficult to detect reductions which occur in the number of microfilariae following treatment with an active drug.

Cotton rats with initial low microfilaria counts (*e.g.*, less than 10 per 100 fields) are not desirable for measuring microfilaricidal activity, since the fluctuations which occur are within the range of comparable non-treated controls.

**DEATH OF ADULT WORMS.** It has been mentioned previously that dead adult worms are occasionally found in non-treated cotton rats, and these may be surrounded by an inflammatory exudate.<sup>6</sup> This does not occur frequently, however, and for the most part living worms only are recovered from non-treated rats. Live worms move actively when placed in 0.8 per cent saline, and continue moving for several hours at room temperature. When observed under a stereoscopic dissecting microscope (x20) the internal structure of living worms is clearly defined, and the outer cuticle is shiny and glistening. Living embryos can be seen within the uteri of mature females under higher magnifications.

Dead worms show no movement when placed in 0.8 per cent saline. They may be clumped or matted, or may be surrounded by an exudate.<sup>7, 8, 12</sup> In later stages of decay, clumped worms may become yellowish and brittle, or may have disintegrated so completely that only fragmentary pieces of cuticle remain. Small or large masses of living worms may be

present in the pleural cavity, or occasionally in the peritoneal cavity, along with one or several clumps of dead worms, after treatment with a filaricidal drug. If animals are held for two or three months following successful treatment, occasionally no worms or macroscopic evidence of their previous presence can be found, in spite of the fact that myriads of microfilariae might have been present in the peripheral blood before treatment was started. We have assumed, in these cases, that the dead worm tissue was completely absorbed.

**THE USE OF CONTROL DRUGS IN SCREENING TESTS.** Although, in many host-parasite relationships used for testing drug activity, control drugs are an essential part of each protocol, they are not essential in the case of cotton rat filariasis, except when different members of the same structural series are being assayed. Sufficient evidence has been accumulated to demonstrate that untreated rats, or rats treated with inactive compounds, rarely demonstrate, upon autopsy, the picture that can be obtained with Neostam, Neostibosan,<sup>7</sup> Stibanosc,<sup>8</sup> the cyanine dyes,<sup>11</sup> or the piperazines.<sup>12</sup>

**In Vitro Studies.** *In vitro* methods, although sometimes giving supplementary data relative to the effectiveness of certain drugs against *Litomosoides carinii*, have not been widely used for screening purposes. Culbertson and Rose<sup>7</sup> transferred macrofilariae aseptically to 50 cc. Erlenmeyer flasks, each containing 10 cc. of a balanced salt solution plus 0.1 per cent glucose, and incubated at 37° C. with or without drugs. Fifty mg. per cent of Neostam killed the worms within twenty-four hours, whereas control worms were active at the end of ninety-six hours. Lower concentrations of the drug killed the worms in correspondingly longer periods of time. The same authors, in cooperation with Hernandez Morales, Oliver-Gonzalez, and Pratt,<sup>10</sup> state that the method did not appear useful for screening drugs, since some compounds were almost inactive *in vitro* but were quite effective in animals and *vice versa*.

Otto and Maren<sup>13</sup> conducted *in vitro* studies with *Dirofilaria immitis*, using phenyl arsenoxides, and found that, although some amide substituted compounds killed the microfilariae at high dilutions, no effect was produced in animals.

Our own experience with piperazine compounds has shown that little correlation exists between their *in vitro* and *in vivo* activity. The cyanine dyes,<sup>11</sup> on the other hand, show activity *in vitro* which corresponds rather highly with the results obtained in cotton rats.

It is apparent, therefore, that for some types of compounds *in vitro* studies may provide useful information regarding their filaricidal properties, whereas in others nothing whatever is gained by this type of study.

**The Use of *Dirofilaria immitis* Infections for Evaluating Filaricides.** The use of dogs infected with heartworm for screening purposes in searching for filaricides is not practical, since their large size limits the number of tests, and the quantity of drug necessary is often

prohibitive. For a long time, however, dogs provided the only available laboratory animal for studying the action of filaricidal compounds, and a large number of antimony and arsenic derivatives have been tested in this host.<sup>15, 16</sup>

Another serious disadvantage in the use of dogs for studies on filaricides is that, frequently, untreated dogs with microfilariae in the peripheral blood reveal no adult worms at autopsy in the heart or lungs. We have experienced this situation several times in both untreated and treated dogs, and have had confirmatory reports from other investigators. It has been disconcerting to treat animals showing relatively high initial microfilaria counts with Fuadin and other compounds, reduce the microfilaria counts, and then at autopsy to find no sign of an adult *Dirofilaria* in any part of the body examined. Dogs with very low microfilaria counts more often than not reveal this condition, at least in so far as our own experience is concerned. It is quite possible that, in dogs receiving an adequate dosage of a filaricidal compound, the adult worms will be killed, will become lodged in the pulmonary artery within the lung, and will there undergo disintegration and eventually complete absorption. Such a process, however, would require a long period of time, and since untreated dogs with circulating microfilariae often reveal no adult worms in the heart or lung at autopsy, this explanation would not be valid in every case.

Adult *Dirofilaria immitis* commonly occur in the right ventricle of the heart, or in the pulmonary artery near the heart. Microfilariae are liberated into the bloodstream and very little periodicity is exhibited. Measured quantities of blood can be withdrawn from the veins in the fore or hind legs, and estimations of the number of microfilariae can then be made per unit sample of blood.

Compounds effective against *Dirofilaria immitis*<sup>13, 16</sup> kill the adult worms, which become lodged in the pulmonary artery within the lung. There is good evidence to show that the simultaneous killing of a large number of worms will produce an embolus in the artery and lead to the death of the animal.<sup>16</sup>

Fuadin<sup>16</sup> is now used by many veterinarians for treating heartworms in dogs, although it is far from being 100 per cent efficient and frequently produces death from toxicity. Otto and Maren<sup>13</sup> have recently obtained very good results in dogs with *p*-[bis-(carboxymethylcapto)-arsino]-benzamide.

The evaluation of effectiveness of treatment with drugs in filaria-infected dogs is more difficult than in cotton rats, particularly if autopsies are not performed. A marked and sustained reduction in microfilariae is not always conclusive evidence that the adult worms have been killed, nor is an improvement in the physical condition of the animal after treatment demonstrative that the worms have been destroyed. We have received dogs for treatment which exhibited the classical symptoms of heartworm infestation; namely, emaciation, shortness of breath after slight exertion, cough, jaundice, and pronounced skin lesions.

Microfilariae of varying quantity occurred in the bloodstream. After providing the animals with good food and adequate care for several weeks, some of them improved markedly in physical condition without treatment. In some cases where the microfilariae were reduced by treatment, and the dogs were then autopsied following a pronounced improvement in physical condition, living worms or no worms whatever were found in the heart or pulmonary artery.

In cases where the microfilariae have been sharply reduced by treatment, and dead adults are found within the terminal branches of the pulmonary artery at autopsy, it can be assumed that a filaricidal effect has been obtained. The reduction of microfilariae *per se* or the absence of adult worms at autopsy are not conclusive evidence that a marked effect has been produced.

**The Correlation Between Filaricidal Effects Obtained in Experimental Animals with Those Obtained in Man.** One of the most important factors in the choice of a laboratory animal for the study of experimental chemotherapy against diseases in general is the correlation which can be expected when clinical trial is given in man. The use of birds and monkeys, for example, in searching for new anti-malarials, has demonstrated that a wide degree of variation can be expected between the effects of drugs against avian, simian, and human malaria. Since the parasites of cotton rat, dog, and human filariasis are not of the same genera, let alone the same species, it would be reasonable to assume that comparable effects with the same drug might not always be produced in the different hosts.

Some correlation has been demonstrated with certain types of compounds between filaricidal action against *Dirofilaria immitis* and *Litomosoides carinii*. Otto and Maren<sup>13</sup> report that *p*-[bis-(carboxymethylmercapto)-arsino]-benzamide kills all adult worms in cotton rats at doses of 4.5 mg. per kg. (0.9 mg. As.) twice daily, intraperitoneally, for six weeks. Intravenous doses of 1.15 mg. per kg. (0.23 mg. As.) once daily for two weeks or longer killed most of the adult worms. In neither host were the microfilariae affected *per se*.

The results obtained with piperazine compounds, particularly 1-Diethylcarbamyl-4-methylpiperazine Hydrochloride, are also somewhat similar in filaria-infected cotton rats and dogs,<sup>12</sup> although a more predictable effect is produced in cotton rats.

Lawton *et al.*<sup>9</sup> found some differences in the effects of several antimony compounds tested in cotton rats and dogs.

The correlation between results obtained in cotton rats and dogs with clinical tests in humans infected with *Wuchereria bancrofti* have thus far not been carried sufficiently far to justify definite conclusions. Brown's<sup>17</sup> results with Anthiomaline in man are suggestive that an effect is obtained similar to that obtained by Brown, Brooks, and Waletsky<sup>18</sup> in dogs. Similarly, the data obtained by Culbertson, Rose, and Oliver-



Gonzalez<sup>19</sup> from treating human filariases with Neostibosan parallel closely the effect of the same compound against *Litomosoides carinii*.<sup>7</sup>

Preliminary results with Hetrazan (1-Diethylcarbamyl-4-methyl-piperazine Hydrochloride) denote a close relationship between filaricidal activity in cotton rats and human cases.<sup>12, 20</sup>

In general, therefore, it appears that data obtained from cotton rats and dogs with regard to filaricidal action are suggestive of similar effects to be produced in human infections. The investigation of a wider variety of chemical compounds may demonstrate some very marked differences in this respect, but at present the choice of the cotton rat for providing leads for the therapeusis of filariasis in man has been amply justified.

**Discussion.** Data which have been published, thus far, relative to the filaricidal action of various types of compounds in cotton rats and dogs have been obtained from naturally acquired infections only. Since the vector of *Litomosoides carinii* in the cotton rat is known,<sup>2</sup> it seems probable that methods will soon be devised for testing the effectiveness of chemical compounds against standardized infections. Scott<sup>5</sup> described a method for producing quantitative infections in cotton rats through the use of the vector (*Liponyssus bacoti*). Bertram, Answorth, and Gordon,<sup>21</sup> and also Hawking and Burroughs,<sup>22</sup> have attempted to transmit the infection to other laboratory animals (albino rats and mice, and hamsters) with some success.

Observations on the rate of growth and maturity in the cotton rat of worms introduced through the vector have been given by Scott,<sup>3</sup> and the pathology of untreated infections is discussed by Wharton.<sup>6</sup>

The use of controlled quantitative infections in any test host for the study of chemotherapy possesses several obvious advantages over naturally acquired infections. The effect of drugs upon different developmental stages of the parasite can be studied, and the prophylactic possibilities of an active compound can be investigated. The relationship between drug dosage and the intensity of the infection can be better evaluated when infections of known character are produced experimentally.

The use of laboratory-produced infections with *Litomosoides carinii* in cotton rats, and possibly in other hosts, should considerably advance our knowledge of the chemotherapy of filariasis in general.

## SUMMARY

Methods used for screening and evaluating the filaricidal action of chemical compounds in cotton rats and dogs are discussed. The advantages of the cotton rat over the dog for screening large numbers of compounds are mentioned, and it is pointed out that thus far the correlation has been good between filaricidal action in cotton rats, dogs and

humans. Possibilities are pointed out for obtaining quantitative evaluations of filaricidal activity in cotton rats through the use of infections induced by the vector.

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## USE OF ARSENICALS IN FILARIASIS\*

By G. F. OTTO AND T. H. MAREN†

*Department of Parasitology, School of Hygiene and Public Health,  
Johns Hopkins University, Baltimore, Maryland*

A careful review of the literature reveals that organic compounds of arsenic were used for filariasis before Rogers (1919) introduced antimony, in the form of sodium antimony tartrate, as a possible therapeutic agent in this disease. There was, apparently, no great enthusiasm over the results obtained, but there has been recurrent use of the arsenicals even since the advent of antimony into this field. Apparently, the only attempt at direct comparison of the filaricidal action of arsenic and antimony with that of other types of compounds was that of Hawking (1940). It is of more than passing interest that all seven of the trivalent types of arsenic compounds which he studied were highly active *in vitro* against the microfilariae of *Wuchereria bancrofti*; three of the seven were more active than any of other 28 compounds including antimony and three more were equaled in 20 hours only by potassium antimony tartrate. However, the same author was unable to obtain any evidence that two of the trivalent types or the one pentavalent arsenical administered to filaria-infected patients had any effect upon these same microfilaria *in vivo*. Apparently, the statement by Goodman and Gilman (1941) that microfilariae "can be killed by organic arsenicals" results from the *in vitro* studies of Hawking despite his failure to show such effects *in vivo*. Most of the clinical trials of arsenic for filariasis have failed to reduce the microfilaria count in either dog or man. However, there are a few reports of direct *in vivo* killing of microfilariae of *Wuchereria bancrofti* by arsenicals. Tanon and Giraud (1921) report that a pentavalent arsenical derived from atoxyl, Hectine (phenylsulfoncamino-phenyl arsenic acid, Raiziss and Gavron, 1923), given in doses of 2 mg. every other day for 20 days eliminated the microfilariac, and that in some cases the microfilariac had disappeared after the third or fifth dose. Ikegami (1920) is reported to have eliminated microfilaria from one patient by two doses of 0.3 g. of arsaminol (Japanese arsphe-amine) and that the patient remained blood-negative for the year he was under observation. Noc (1923) reports finding only dead and shriveled microfilariae in the blood of patients following the injection of eparseno (apparently a trivalent type of arsenical, the diglucoside of

\* This work was carried on initially under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University; it was continued under a contract between the Office of the Surgeon General, U.S.A., and the same university.

† Now in the Department of Pharmacology and Experimental Therapeutics, School of Medicine, The Johns Hopkins University.

arsphenamine). More recently, Brown and Schoenbach have each reported the elimination of microfilariae from a patient receiving the intensified course of mapharsen (the latter author used the 5-day constant drip method), and Rose and Culbertson (1945) found that the microfilariae dropped six to seven months after the daily administration of melarsen oxide in doses, which produced encephalitis in 2 of the 18 patients they treated.

There was, apparently, no direct evidence that the adult worms had been injured either *in vivo* or *in vitro* by the arsenic in these earlier studies. It is of interest, however, that Tanon and Giraud found that the microfilaria did not recur within the two to three months of observation following the use of Hectine and that the patients were clinically improved; specifically, the chyluria had disappeared and the total and differential leucocyte counts returned to normal. Likewise, Ikegami's case treated with arsphenamine remained microfilaria-free for a year and the chyluria which had cleared up immediately after treatment did not recur. It may well be that the adult worms were destroyed in these cases. Likewise, the loss of microfilaria over a six- to seven-month period after the administration of melarsen oxide by Rose and Culbertson may perhaps be due to the death of the adult worms. A number of other workers have reported clinical improvement without demonstrable destruction of the microfilaria. Thus, Chopra and Rao (1929) noted that the use of a pentavalent arsenical, tryparsamide, reduced chyluria and eliminated the microfilaria from the urine. The same authors (1939) reported the same results with another similar compound, Soamin (atoxyl) and, further, that there was general clinical improvement. They concluded that Soamin was the best drug available for "controlling the infection in its early stages." However, because it did not reduce the microfilaria count, it was placed second to Fuadin, which produced a temporary reduction in the microfilaria, though usually without clinical improvement. Van der Sar and Hartz (1945) likewise reported clinical improvement with reduction of eosinophilia, following the use of mafarside (mapharsen). Various explanations have been offered for this clinical improvement. O'Connor (1922) thought it might result from the rest imposed during the treatment. Others have referred to the "tonic" effect of the arsenic; among these is Hays (1933), who reported clinical improvement following the administration of arsphenamine to dogs infected with the heartworm, *Dirofilaria immitis*. None seem to have seriously considered that such improvement might have been brought about by the death of the adult worms. On the other hand, King (1944) noted the acute exacerbation of lymphangitis and epididymitis following the use of mapharsen and raised the question as to whether or not this may have resulted from worms killed *in situ* by the treatment. The data are rather meager and few cases have been followed long enough to determine whether the benefits are temporary or may be considered to have been permanent.

Thus, previous work, although fragmentary and inadequate, has established (1) that the trivalent type arsenic compounds have an *in vitro* microfilaricidal action; (2) that with intensified therapy some of them, at least, have an *in vivo* microfilaricidal action; and (3) that there is some indirect evidence that adult worms may have been killed or injured by the intensified therapy. In view of our own work with arsenic and antimony (Otto and Maren, 1947; Otto, Maren, and Brown, 1947; Otto and Maren, in press) and recent reports in the literature (*viz.*, Brown and Thetford, 1946; Culbertson, Rose, and Oliver-Gonzalez, 1945) we raise the question whether or not the negative results obtained by many workers with arsenic given at weekly intervals or twice a week have any real significance, and it is, perhaps, surprising that no one seems to have given arsenic a more thorough study in this connection. It would be very comforting to be able to say that our own interest in the filaricidal possibilities of arsenic was a logical development from this review of the literature. Such, however, is not the case; it was only after we had rediscovered the filaricidal action of this substance that a most careful review of the literature revealed the earlier reports.

When our studies were initiated, in 1943, we directed our attention, in common with most others, towards antimony, but we early became convinced that antimony offered little opportunity for an improved therapy of filariasis. In considering the possibilities in other types of compounds, our interest was directed most sharply to the rather extensive field of non-metallic organic compounds. Accordingly, it was almost by accident that, early in our studies, mapharsen was included with a group of compounds for *in vitro* assay.

## MATERIALS AND METHODS

No attempt will be made, here, to outline in detail the procedures of our study. Initial screening of drugs was *in vitro*, utilizing the microfilaria of *Dirofilaria immitis* as the test organism in many cases compared directly *in vitro* against the adults of *Litomosoides carinii*. This was followed by *in vivo* assays, in *L. carinii* infected cotton rats, of compounds selected after *in vitro* trial. No compound which offered any promise was discarded unless it failed to kill adults of *L. carinii* after 40-45 daily doses at a level, as near as we could judge, close to the maximum tolerated dose; in some cases this actually exceeded the L.D.<sub>50</sub>. Compounds which survived this test were administered to cotton rats in various reduced schedules and acute toxicity determined on mice if adequate information was not available. As a result of these studies, a selected few compounds were administered to dogs first in the 40-45 day schedule at toxic levels and, if justified, thereafter in reduced schedules. Although microfilarial counts were made, the criterion of effectiveness was death of the adult worms. Two arsenicals which survived these tests were subjected to a thorough study of their pharmacological properties including blood levels,

excretion rates, retention in tissues, and tolerance in a number of species of laboratory animals. One of these was finally administered to filaria-infected patients.

## DISCUSSION OF RESULTS

As indicated above, mapharsen was, more or less accidentally, run through our *in vitro* screening procedure and proved to be much more active than any of the antimony compounds and the approximately 75 non-metallic compounds we had tested by that time. Because of our knowledge of its use in man for syphilis, and despite previous reports of its failure to reduce the microfilaria counts, it was immediately administered to *L. carinii* infected cotton rats in doses of 0.9 mg. As. (3.0 mg. mapharsen)/kg. b.i.d. for 45 days and to a *D. immitis* infected dog in doses of 0.6 mg. As. (2.0 mg. mapharsen)/kg. daily for 35 days. The results were completely negative, so that rather than consider this drug further we intensified our consideration of other substituted phenyl arsenoxides. The pentavalent analogues of some of them were also studied. We are indebted to Dr. Harry Eagle of the United States Public Health Service, the National Research Council, and various commercial concerns who kindly supplied many of these arsenicals.

We may dismiss the pentavalent arsenicals briefly with the note that all of those considered were either inactive at any dose both *in vitro* against microfilaria of *D. immitis* and *in vivo* against *L. carinii* in the cotton rat, or were active only in highly toxic doses, usually at or above the L.D.<sub>50</sub> level.

It may be well to note that the phenyl arsenoxides were all compared on the basis of arsenic equivalents, and when compared directly with trivalent antimony compounds, in the latter part of our work, this comparison was on the basis of equivalent moles of the two metals and not on the basis of equivalent weights either of metal or total compounds. Incidentally, if the comparison on the basis of equivalent moles of metal alters the picture, it gives antimony a more favorable place in the comparison of filaricidal activity than if it had been on the basis of equivalent weights of metal. All of the phenyl arsenoxides were much more active *in vitro* than antimony or any of the non-metallic compounds tested: not only did arsenic kill at higher dilutions, but even at killing dilutions its action was much more rapid than that of antimony. There was, however, about a 30-fold difference between the least active and the most active of these phenyl arsenoxides, while the differences in toxicity for mice was scarcely more than three-fold.

The most active were the amide-substituted compounds, either the *p*-arsenosobenzamide or the *p*-arsenosobenzenesulfonamide types, and these compounds had a favorable therapeutic index as judged by microfilaricidal activity *in vitro* and acute L.D.<sub>50</sub> in mice. The administration of the phenyl arsenoxides to cotton rats in doses of 0.9 mg. As/kg. b.i.d. for 25 and 45 days again singled out the amide-substituted compounds;

they alone reduced the microfilaria blood level 70-90 per cent during treatment and killed 90-100 per cent of the adult worms. The administration of these same compounds at half the above dose for the same period of time, and various other reduced doses, singled out *p*-arsenosobenzamide (T.D.C. #622) as offering the greatest promise. At these reduced doses, however, it had a lethal effect upon the adults without killing the microfilariae. Accordingly, it was administered to *D. immitis* infected dogs, and toxicity and pharmacology were studied in a variety of laboratory animals.

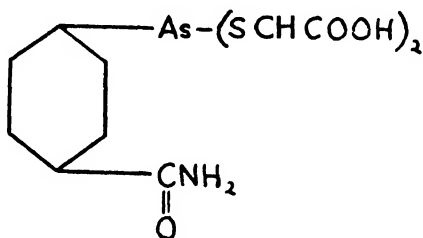
Briefly, the results of the pharmacological studies revealed that it had about the same degree of both acute and chronic toxicity as mapharsen. There was no appreciable concentration of the arsenic in any particular organ, but that portion not immediately excreted was more or less evenly distributed through all tissues studied. One conspicuous feature of the arsenic distribution was that the adult worms invariably contained as much metal as the host organs, and usually they contained much more. The compound was easily tolerated by cotton rats in intraperitoneal doses of 0.9 mg. As/kg. b.i.d. for 45 days and by dogs in intravenous doses of 0.9 mg. As/kg. daily for the same length of time. Doses as low as 0.45 mg. As/kg. for 37 and 45 days killed all the adult worms in the dog. It may be worth noting, here, that these dead worms were found in the pulmonary arteries, often deep in the lungs, and only in rare instances were any of them found in the right ventricle of the heart which is their normal habitat when alive.

Reduced doses, as in the cotton rat, failed to effect the blood microfilaria level. Furthermore, the drug could scarcely be considered for routine administration in either water or saline since it is very insoluble (1:800). Despite these two undesirable features, the compound seemed to offer some promise since, so far as we know, it was the only one known to kill all the adult filaria in both the cotton rat and the dog in doses which even approached that which might be considered as feasible in routine practice in either human or veterinary medicine. Thus, considerable thought was given to the question of increasing its solubility before proceeding with a more detailed study of its therapeutic efficiency at lower doses. We discussed at considerable length the possible use of organic solvents as the vehicle for its administration, but this possible procedure was discarded without experimentation; rather, attempts were made to increase the solubility without materially altering toxicity or therapeutic effectiveness by changes in the chemical structure of the compound itself. Accordingly, three other compounds were obtained, two of them by synthesis in our laboratory, in which the oxygen of *p*-arsenosobenzamide was replaced by organic radicals. These compounds were produced by replacing the oxygen with dicysteinyl, dithio-salicylic, and dithioglycollate respectively. It is not necessary, here, to make a detailed comparison of these compounds. Considerations of tox-



icity, therapeutic action, and solubility focused our attention on the last-named.

This compound is *p*-[bis-(carboxymethylmercapto)-arsino]-benzamide and now bears the non-proprietary name, arsenamide, approved by the Council on Pharmacy and Chemistry of the American Medical Association (1948). It has also been referred to in the literature under Tropical Disease Center #970 and as the thioarsenite of *p*-arsenosobenzamide (Otto and Maren, 1945). Its structural formula is as follows:



Maren (1945) has reported on the chemistry and synthesis of arsenamide.\* It is a white powder containing 20 per cent arsenic and stable at room temperature; the sodium salt is readily soluble; and it is stable both in buffered and unbuffered solutions in amber ampoules at either refrigerator or room temperature. We have used it intravenously in 2 per cent solutions as the sodium salt both in buffered (pH-6.9-7.1) and in unbuffered solution (pH 5.9-6.1).

Preliminary *in vitro* and *in vivo* studies indicated that, in terms of arsenic equivalents, arsenamide had essentially the same therapeutic possibilities as its precursor, *p*-arsenosobenzamide, and had essentially the same acute L.D.<sub>50</sub> for mice as both *p*-arsenosobenzamide and mapharsen. Accordingly, in order to obtain further information on its therapeutic action and its toxicity for animals, it was administered in graded daily intravenous doses to a series of dogs (TABLE 1, reproduced from Otto and Maren, 1947). Both dogs (#11 and #25) receiving daily doses of 1.8 mg. As/kg. died on the fifth day, one of them having received only 3 doses. However, the adult filariae had already been injured, since the only three worms in one dog and five of the 38 in the other were dead, while the remaining were very inactive. Some of them responded only very sluggishly to tactile stimuli, both immediately upon removal from the dog and after two and 24 hours incubation at 37° C. The remaining dogs, even those (#5 and #27) receiving 0.9 mg. As/kg. daily for 30 days, suffered no demonstrable ill effects. Attention is accordingly directed towards dog Number 31, which died on the 12th day after only

\* We are indebted to C. K. Banks, of Parke, Davis and Company, Detroit, who later supplied the main stock and its precursor (#622) from which we made much of our supply; to Fitzgerald Dunning and J. H. Brewer, of Hynson, Westcott, and Dunning, Baltimore, who ampouled the compound in 2 per cent solution for use; and to C. K. Banks and A. C. Bratton, Jr., who ampouled our more recent supply of the 2 per cent solution.

TABLE 1  
TREATMENT OF *Dirofilaria immitis* WITH DAILY INJECTIONS OF  
ARSENAMIDE (T.D.C. #970)

Dog	Daily dose (mg. As/kg.)	No. of injections	Total dose (mg. As/kg.)	Fate of dog	Adult worms	
					Alive in heart	Dead in pulmonary artery
11	1.8	5	9	D, 5 days	33 (Sluggish)	5
25	1.8	3	5.4	D, 5 days	0	3
5	0.9	30	27	Healthy, K	0	11 ♀ 11 fr.
27	0.9	30	27	Healthy, K	0	2 fr.
28	0.9	15	13.5	Healthy, K	0	7 ♂ 2 ♂ 2 fr.
9	0.9	15 (alternate days)	13.5	Healthy, K	1 ♀ (Sluggish)	0
29	0.45	15	6.8	Healthy, K	0	3 ♀ 2 fr.
32	0.23	15	3.4	Healthy, K	0	4 ♀ 6 ♂ 2 fr.
31	0.23	11	2.5	D,* 12 days	0	19 ♀ 12 ♂
34	0.115	15	1.7	Healthy, K	1 ♂; 2 ♀ (Active)	0

\* This dog was critically ill before experiment started.

fr. = fragmented.

D, Died; K, Killed.

11 daily injections 0.23 mg. As/kg.; this dog was critically ill, had refused food, and seemed moribund before treatment was initiated. It was used because it was the only dog available and we were rather surprised that it survived long enough to receive 11 injections. It will be noted that all worms present were dead in all dogs receiving daily injections of 11 doses or more down to and including this animal. It would appear that the effectiveness of the drug may not be measured in terms of the total dose, since the worms were not all dead in the animal (#11) receiving a total of 9.0 mg. As/kg. over a five-day period and the one (#9) receiving 13.5 mg. in doses of 0.9 mg. every day over a 30-day period, whereas they were all dead in dogs 31, 32, and 29, receiving totals of only 2.5 to 6.8 mg. As/kg. in daily doses over an 11- to 15-day period. While the data are obviously not conclusive, they have the support of similar data in the cotton rat not only with arsenamide but with other of the phenyl arsenoxides as well. Although it is obvious that the dose schedule cannot rest alone on the most effective therapeutic procedure but must be reconciled with considerations of toxicity as well, the point, nevertheless, has some importance and will warrant further investigation.

The only worms in the treated dogs which were comparable in activity to those in untreated animals were those in the animal (#34) which received the lowest dose (0.115 mg. As/kg. daily for 15 days). It seems evident, then, that the minimum effective dose apparently is somewhere near the level of 0.2-0.23 mg. As/kg. daily for two weeks.

Essentially all of the above data were obtained more than two years ago and we were tempted, at that time—indeed, we were under some pressure—to give this compound clinical trial in man. Although it seems obvious that some risk is involved in the administration of any therapeutic agent to man for the first time, we are of the persuasion that these risks may be reduced to a really unavoidable minimum by adequate study of the basic pharmacology in a variety of laboratory animals. Accordingly, the acute toxicity was further investigated, but particular attention was given to the effect of daily doses repeated over a long period of time, in a variety of laboratory animals including the monkey. These studies involved excretion rates, blood levels (including plasma-cell partitions), and tissue concentrations after multiple daily injections. Attention was also given to the gross and microscopical anatomy after fatal as well as after long sustained readily tolerated doses.

It will be sufficient, for the purposes of this review, to comment briefly on the results in monkeys. Seven *Rhesus* monkeys were given a graded series of 20 intravenous injections over a 21-day period (TABLE 2).

TABLE 2  
RESULTS OF REPEATED INTRAVENOUS DOSES OF ARSENAIDE  
IN *Rhesus* MONKEYS

Number	Weight (kg.)	Daily dose mg. As/kg.	No. doses	Fate
M 821	2.7	0.3	20	Survived, healthy
M 817	3.2	0.45	20	Survived, healthy
M 824	2.2	0.9	20	Survived, healthy
M 819	2.8	0.9	20	Survived, healthy
M 830	2.6	1.35	20	Survived, healthy
M 827	1.8	1.35	8	Died 5 hrs. after 8th inj.
M 818	3.1	1.8	20	Survived, healthy

Since it was visualized that the preliminary clinical trial in man might well be at the level of 0.3 mg. As/kg. for 15 days, the lowest dose administered to monkeys was at this daily dose level, but the total was extended by a third to 20 doses. It will be noted that the only fatality was one of the two monkeys receiving 1.35 mg. As/kg. However, none of the other monkeys showed any ill effects, including the other animal on this same dose and the one receiving 1.8 mg. There was no loss of appetite, activity, or weight; in fact, three of the animals, including these last two, gained about half a kg. It is interesting to note that only the lightest monkey (1.8 kg.) died, but one hesitates to attach too much significance to the fact. The six surviving monkeys were killed 48 hours

after the last injection, and arsenic determinations were made on various organs by Maren's (1946) modification of Magnuson and Watson's method. There was no discernible gross pathology and, so far, we have been unable to find any evidence of microscopical pathology, but this is being re-checked by the pathologist. At the lowest doses, there was no conspicuous accumulation of arsenic in the internal organs, with the possible exception of the thyroid, which contained 4 to 8 micrograms per gram, and the liver which contained 1 to 2 micrograms per gram. In the monkeys which survived the higher doses (0.9-1.8 mg.), the thyroid contained 12 to 26 micrograms per gram, but the liver content was no higher than at the lower doses. The monkey (#827) which died 5 hours after only eight doses of 1.35 mg. As/kg. had conspicuously more arsenic in most organs except the thyroid gland. Thus, the lung contained 2.2, the spleen 2.3, and the liver 21.0 mg. As/g. It seems likely that the short interval between the last injection and death, as well as the fact that the animal was not able to handle the arsenic as well as the others, may account for these unusually high values.

Arsenic levels in both blood cells and blood plasma were followed during the first 48 hours after the initial injection and again after the final injection. There was essentially no difference in the blood levels at the beginning and at the end of treatment. In both cases, blood levels were variable within the first hour after injection and tended to level off at less than 0.1 microgram per gram, in some cases remaining near that level for 48 hours but in others dropping within the first 24 hours. In general, the cells contained slightly more arsenic than did the plasma, but there was no conspicuous movement of the metal to the cells such as occurs with unsubstituted phenyl arsenoxide (Hogan and Eagle, 1944) or with trivalent antimonials (Otto, Maren, and Brown, 1947).

Urinary excretion of the arsenic amounted to 7 to 10 per cent during the first 48 hours after the initial injection and 5.5 to 11 per cent during the same period after the last injection. Fecal excretion during the same period amounted to 1 to 7 per cent after the initial injection and 2.5 to 13 per cent after the last.

As a result of these studies on monkeys and collateral studies on other laboratory animals, it seemed reasonable to administer this chemical to man in daily doses of 0.3 mg. As/kg., but we finally decided to give preliminary clinical trial with 0.2 mg. As/kg. daily for 15 days, since this approximates the minimum effective dose in dogs. Accordingly, the drug was administered in this dose schedule to 18 negro adults harboring microfilaria-positive but asymptomatic *Wuchereria bancrofti* infections in St. Croix, Virgin Islands.\* The treatment was easily tolerated. Nine of the patients became blood-negative, as judged by the examination of 0.1 cc. of night blood. The microfilaria count dropped nearly to zero in the remaining nine: by way of example, one dropped from 760 to 2.

\* These studies were carried out jointly with Doctors H. W. Brown, N. D. Thetford, and S. D. Bell, Jr., and were supported in part by a grant from the John and Mary R. Markle Foundation to the School of Public Health, Columbia University.

Six of these patients have now been followed five to ten months post-treatment and there have been no changes in the microfilaria level except that two of those which were not completely free of the microfilaria immediately after treatment failed to show microfilariae on the last examination; one of those previously reported negative has since shown one microfilaria by Knott's (1939) method applied to 1.0 cc. of blood (Thetford, Otto, Brown, and Maren, 1948). Thus, in so far as the microfilaria count may be taken as an index of cure, there were no complete failures, and half of those treated would, on this basis, be classified as cured.

It is more interesting to note that this chemical, which did not destroy the microfilariae of either *Litomosoides carinii* or *Dirofilaria immitis* when administered to the host in even higher doses, quickly destroyed those of *Wuchereria bancrofti*. This raises a number of important questions. Is the latter species more susceptible than the two former? Are there any differences in the physiological reaction of the hosts which are of significance? Is it possible that there is complete reversal of the action so that the adult worms are uninjured or at least not killed while the microfilariae are destroyed in man, whereas the reverse is true in the dog and cotton rat? These are some of the questions which remain to be answered with reference to this compound. It would appear, however, since there was no return of microfilariae in those patients following six to eight post-treatments, that the adult worms had suffered at least some injury. We have no evidence that any of these phenyl arsenoxides sterilize the adult females of either *L. carinii* or *D. immitis*, such as appears to be the case with most of the trivalent antomoniols (Ashburn, Perrin, Brady, and Lawton, 1945). However, we have not, as yet, explored this possibility sufficiently to be able to say definitely that such action does not occur. There is some suggestion that at least some of the adults may have been killed, since some of the patients suffered acute attacks of lymphangitis or lymphadenitis in the inguinal region. More studies are needed before we can be certain of the full significance of these observations. At present, all we can say with assurance is that arsenamide destroys the microfilariae of *W. bancrofti* at the same dose which has been shown to kill the adult filariae (*D. immitis*) in the dog; so far as we can determine from the literature, this is the first and only time this relationship has been reported. It seems evident, then, that until even more favorable therapeutic agents are available the substituted phenyl arsenoxides warrant serious consideration in the therapy of filariasis, and that further study of arsenamide is justified.

### SUMMARY

The literature and our own studies indicate that the trivalent type of organic arsenicals are active in both canine and human filariasis, and the substituted phenyl arsenoxides are singled out as warranting particular consideration. One of these, *p*-[bis-(carboxymethylmercapto)-

arsino]-benzamide, now known under the non-proprietary name of arsenamide, has been shown to kill the adults of *Dirofilaria immitis* in dogs in easily tolerated doses. This same dose is easily tolerated by man and has been shown to destroy the microfilaria of *Wuchereria bancrofti*. So far as we know, this is the only therapeutic agent for which both of these observations have been reported. It remains to be determined whether or not the adults of *W. bancrofti* are killed by this treatment.

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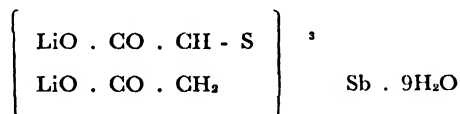
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# TREATMENT OF FILARIASIS WITH ANTHIOMALINE (LITHIUM ANTIMONY THIOMALATE)\*

By H. W. BROWN

*School of Public Health, Faculty of Medicine,  
Columbia University, New York, N. Y.*

**Introduction.** The development of our knowledge concerning the therapy of filarial infections in man has been marked by extremely fruitful results in the past ten years. The extent of our knowledge in 1937 was that several antimony and arsenical compounds would temporarily depress the microfilaria counts in man, but a drug that would give a permanent reduction was unknown. Today, there are a number of drugs that give promise of curing filarial infections. The relative success of Fuadin in *Dirofilaria immitis* infections naturally directed attention to the whole antimony-containing group. We began with a study of the effectiveness of Fuadin in canine filarial infections in 1938, and also used it in conjunction with the (then) new drug, sulfanilamide (Brown,<sup>1</sup> Brown and Sheldon<sup>2</sup>). The shortcomings of Fuadin led us to try other antimony compounds, among them anthiomaline, lithium thiomalate. Anthiomaline has the following structural formula:



It is supplied as a 6 per cent solution, 1 cc. containing 60 mg. of anthiomaline or 10 mg. of antimony.

When our program of filaria therapy was begun, the dog heartworm, *Dirofilaria immitis*, was the only parasite available for experimentation. We proceeded, therefore, to test the efficacy of anthiomaline in infected dogs. Although practically all therapy using antimony compounds was given on alternate days or with even longer lapses, the advantages of a short course of therapy were so apparent that we tried the effect of daily doses instead.

Nine to eleven daily doses of anthiomaline containing from .80 to 2.10 mg. of antimony per kg. of animal weight given intramuscularly, promptly reduced the microfilaria count 95, 100 and 100 per cent in three dogs (TABLE 1). Dog No. 2 died, however, and toxicity studies in progress with

\* The financial support of the John and Mary R. Markle Foundation is gratefully acknowledged. The anthiomaline was kindly furnished by Merck and Company, Rahway, N. J.  
Some of the data presented here are part of unpublished studies made with Drs. T. J. Brooks, Jr., E. Waletzky, and G. Spremull.



TABLE 1

TREATMENT OF *Dirofilaria immitis* WITH REPEATED DAILY INTRAMUSCULAR INJECTIONS OF ANTHIOMALINE\*

Day	Dog #1 12.5 kg.		Dog #2 9.5 kg.		Dog #3 13.5 kg.	
	Dose as mg. Sb/kg. wt. dog	Micro-filariae per cc.	Dose as mg. Sb/kg. wt. dog	Micro-filariae per cc.	Dose as mg. Sb/kg. wt. dog	Micro-filariae per cc.
1	0.8	390	1.05	200	.74	210
2	0.8	180	1.05	180	.74	220
3	0.8	200	1.05	150	.74	160
4	0.8	220	1.05	130	.74	140
5	1.2	190	1.57	100	1.11	150
6	1.6	180	2.10	80	1.48	100
7	1.6	120	2.10	70	1.48	70
8	1.6	110	2.10	10	1.48	40
9	1.6	50	2.10	0	1.48	0
10	1.6	20	2.10	0		0
11	1.6	30		0		0
12		10		Died		0
13		20				0
14		40				0
15		20				0

\* From the thesis of T. J. Brooks, Jr., University of North Carolina, 1912.

the therapeutic tests suggested that the dosage, although therapeutically active, was well into the toxic level. Lawton, Brady, Ness, and Haskins<sup>3</sup> report that six intravenous injections of anthiomaline at 0.8 mg. Sb/kg. of body weight eliminated the circulating microfilariae from their experimental dogs.

TABLE 2

EFFECT ON MICROFILARIA COUNT OF *Dirofilaria immitis* OF ANTHIOMALINE GIVEN INTRAMUSCULARLY

Dog No.	Wt. kg.	Anthiomaline mg. antimony per kg. dog	Number doses	Microfilariae per cc. blood		Days followed
				Before treatment	After treatment	
11	10.5	4.76	1	20	0	8
12	21.	4.70	1	5,000	0	515
10	12.5	4.0	1	1,620	0	7
7	16.	3.1	1	150	28	18
51	11.6	4.0	2	430	50	39
44	11.6	3.5	2	360	10	90
31	20.6	3.0	2	170	0	60
60	11.4	2.5	2	35	10	60
14	15.0	3.0	2	50	60	44
5	12.3	3.4	3	32,280	0	110
4	18.	2.2	3	80	0	died-1
62	6.9	2.0	3	115	0	60
84	5.9	2.5	3	230	0	15
13	15.7	2.3-3.0	4	60	0	157

Since the repeated daily doses of anthiomaline in our experience did not free the bloodstream of all microfilariae, and since the drug's toxicity is due to cumulative effects, we next studied the effects of from 1 to 3 large doses given intramuscularly. Three of four dogs (TABLE 2) given a single injection of 4.0 to 4.7 mg. of antimony per kg. of weight were all microfilaria-negative in from 3 to 7 days. Dog No. 12, whose original count was 5,000 microfilariae per cc., was still blood-negative after 515 days. The other 2 dogs were autopsied 7 and 8 days after their blood became free of microfilariae.

The fourth dog, No. 51, exhibited a drop in microfilaria count from 430 to 50 per cc. of blood. After 30 days, a second dose at the rate of 4.0 mg. antimony per kilogram of body weight was given, yet the microfilaria count persisted at 50 per cc. One dog given a single intramuscular injection of 3.1 mg. of antimony per kilogram experienced an 81 per cent drop in microfilaria count.

These preliminary results using a large single dose of anthiomaline were encouraging until a series of 8 dogs were given similar doses for toxicity studies and two of the dogs (25 per cent) died.

In view of the toxicity of the single large dose of anthiomaline, we attempted a compromise between repeated small doses and a single large dose. Additional dogs were treated with from 2 to 4 daily injections of smaller amounts of the drug (TABLE 2). Two injections of 2.5 to 3.5 mg. of antimony per kilogram reduced the microfilaria count to 0 in only one of four dogs, and one of the four dogs died as a result of the treatment. Three doses of 3.4 mg. of antimony per kilogram of dog were well tolerated by a single dog, whose microfilaria count was immediately reduced from 32,280 per cc. to 0, where it remained during the 110 days of observation. Three other dogs given 2.0 to 2.5 mg. of antimony for three consecutive days all became microfilaria-free, yet the dog on the 2.2 mg. dose died one day after completion of treatment. Studies are still in progress to ascertain whether or not there is a safe effective dose of anthiomaline for use against *Dirofilaria*.

The test of effectiveness of anthiomaline against *D. immitis* has been given above in terms of microfilaria reduction, although it is apparent that the adult worm residing in the heart cavity is the real object of the therapeutic tests. A number of the dogs were autopsied from 2 to 30 days after completion of the various anthiomaline treatments and a search was made for the adult worms. In several instances, live sterile worms and live adults that passed microfilariae when placed in saline were found. Disappointingly few dead worms were found. In a number of instances, no adult worms were found. Steele<sup>5</sup> reported, however, that two dogs treated with anthiomaline at the Naval Medical Research Institute became blood-negative for microfilariae and four months after treatment autopsy disclosed only dead adults.

Monosodium antimony thioglycollate and Fuadin, both trivalent drugs, similarly clear the blood stream of *Dirofilaria* microfilariae, yet leave living adults in the heart.

TABLE 5  
EFFECT OF ANTHIOMALINE *in vivo* ON *Litomosoides carinii* ADULTS  
AND MICROFILARIAE  
(Bieter;<sup>6</sup> Culbertson;<sup>7</sup> Robinson<sup>8</sup>)

Total dosage mg. of antimony per kg. of rat	Route	Duration of dosage days	% Reduction in microfilariae	% Adults dead at autopsy
(B) 43.2	i.p.	6	0	0-25
(B) 86.4	i.p.	12	0	50-100
(C) 50.0	i.m.	30	0	0
(R) 1020.0	i.m.	119	99-100	100

that of *D. immitis* inhabiting the cavity of the dog's heart. TABLE 5 gives the results of studies by Bieter,<sup>6</sup> Culbertson,<sup>7</sup> and Robinson,<sup>8</sup> using anthiomaline against *L. carinii*. Bieter found that 86.4 mg. of antimony per kg. of rat given intraperitoneally over 12 days killed 50-100 per cent of the adult worms but did not reduce the microfilaria count. These results indicate that the adult worms are more susceptible to anthiomaline than are the microfilariae. On the other hand, Robinson, who gave 20 mg./kg. intramuscularly three times a week for 17 weeks, reports both the adults and microfilariae killed. At the end of 13 days, intramuscular therapy, total antimony 100 mg./kg., he noted a 68 per cent reduction in microfilaria count, whereas Bieter after 12 days of intraperitoneal therapy with a total of 86.4 mg./kg. of antimony found no reduction in microfilaria count. These doses are so nearly similar that one suspects that the route of administration may have been responsible for the great difference in effect.

***In Vitro* Tests against Microfilariae.** As part of screening tests for new filaricidal drugs, *in vitro* experiments using microfilariae of *D. immitis*, *W. bancrofti*, and *L. carinii* have been used. Although the shortcomings of such a test are well known, it has the advantage of simplicity and may be of value especially in ascertaining the relative activity of members of a chemical series. We used the *in vitro* test to compare the activity of anthiomaline against the microfilariae of *D. immitis* and *W. bancrofti* in order to get a comparison of its possible activity against them in their respective hosts. The test is simple; microfilariae are centrifuged from infected blood and placed in a saline solution containing varying concentrations of the drug. After two and a half hours and twenty-one hours of contact with the drug in a 30° F. incubator, a count is made under the microscope to ascertain the percentage of microfilariae that have been killed. TABLE 6 gives the result of such a test. It will be seen that the two and a half hours of exposure to various concentrations of anthiomaline had little effect on the microfilariae of either species. After exposure for twenty-one hours, however, 100 per cent of the *W. bancrofti* microfilariae were dead in both the 1-1,000 and 1-10,000 dilutions of the drug. The activity against *D. immitis* microfilariae was slightly less. These findings

TABLE 6

*In vitro* TESTS OF ANTHIOMALINE AGAINST *Wuchereria bancrofti* AND *Dirofilaria immitis* MICROFILARIAE IN TERMS OF PERCENTAGE OF LARVAE KILLED BY EXPOSURE TO DIFFERENT CONCENTRATIONS OF THE DRUG

Hours exposure to drug	Parasite	Dilution of anthiomaline in Locke's solution			
		1-1,000	1-10,000	1-100,000	1-1,000,000
2½	<i>W. bancrofti</i> <i>D. immitis</i>	0 0-2	0 0-13	0	
21	<i>W. bancrofti</i> <i>D. immitis</i>	100 80-100	100 56-80	1-33	0

were confirmed *in vivo*, as the microfilariae of *W. bancrofti* disappear from man's blood after smaller per kilogram doses than do the microfilariae of *D. immitis* of the dog.

**Anthiomaline Blood Levels and Excretion Rates.** Although antimony has been used for many years in the treatment of tropical diseases, there are still relatively few data available concerning its disposal within the body. The development in 1943, by Goodwin and Page,<sup>9</sup> of a polarographic method, and in 1945 by Maren,<sup>10</sup> of a colorimetric method, has made possible accurate investigations of the distribution and excretion of antimony in the body. Otto, Maren, and Brown<sup>11</sup> studied the antimony

TABLE 7

ANTIMONY LEVELS IN MICROGRAMS PER GRAM OF CELLS AND OF PLASMA AT INTERVALS (HOURS) AFTER INTRAMUSCULAR INJECTION OF 0.5 MILLIGRAM OF Sb PER KILOGRAM AS TRIVALENT ANTIMONY

(After Otto, Maren, & Brown<sup>11</sup>)

	Patient	Time (hours)							
		0.25	0.5	1	2	3	6	12	24
Blood cell levels	J.W.	.15	.40	.67	.55	.50	0.14	0.10	0.13
	J.H.	.11	.31	.63	.70	.70	0.19	0.12	±
	R.O.	.47	.93	.56		.87	0.7	0.3	0.07
	A.W.	.31	.44	.67	.57	.64	0.33	0.22	0.21
	B.G.	.45	.87	1.46	1.20	.87	0.42	0.32	0.21
	P.R.	.14	.40	.68	.77	.53	0.36	0.37	0.26
	Averages	.26	.56	.78	.76	.68	.36	.19	.15
Blood plasma levels	J.W.	.12	.21	.17	.16	.13	0.10	0.08	0
	J.H.	.09	.17	.16	.13	.11	0	0	0.11
	R.O.	.09	.09	.17		.20	0.13	0.17	0.08
	A.W.	.19	.23	.14	.14	.12	0.09	0	0
	B.G.	.17	.18	.21	.08	.13	0.24	0.21	0
	P.R.	.09	.12	.20	.10	.10	0.09	0.18	0
	Averages	.13	.17	.18	.12	.13	.11	.11	.03
Cell/plasma ratio		2.0	3.3	4.3	6.3	5.2	3.3	1.7	5.0

blood levels and excretion rates of a number of individuals under treatment for filariasis with organic trivalent and pentavalent antimony compounds. Anthiomaline was injected intramuscularly daily at a rate of 0.5 milligrams of antimony per kilogram of body weight. The distribution of antimony in the blood was characterized by the rapid movement of the metal to the cells and by the higher concentration in the blood cells than in the plasma. As early as 15 minutes after the initial intramuscular injection of anthiomaline, the antimony concentration of the cells was, on the average, twice that of the plasma (TABLE 7, and FIGURE 1). The cell content increased rapidly within the next hour, while there was only a slight increase in the plasma content. On the average, the plasma content fell off after an hour and remained constant for 1 to 2 hours at approximately the level found at the 15-minute determinations. No antimony was detected at 24

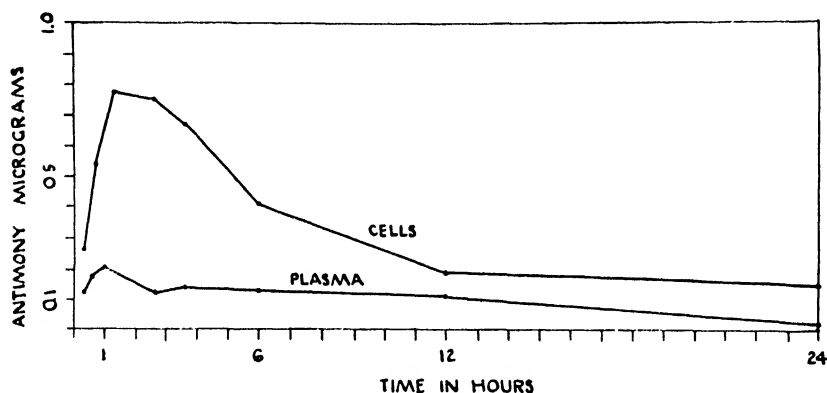


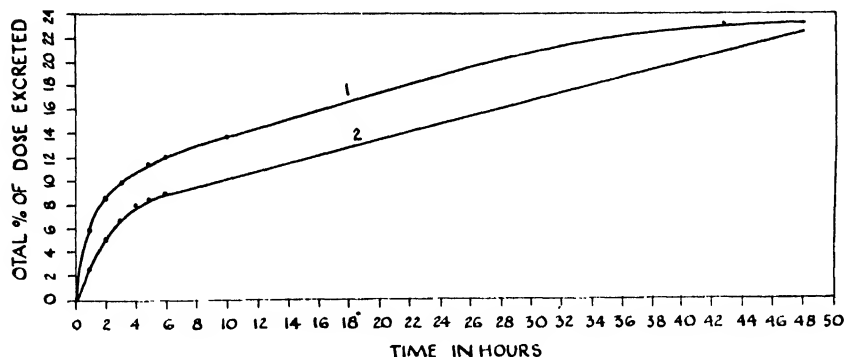
FIGURE 1. Antimony levels (in micrograms of Sb per gram) in blood cells and plasma during the first 24 hours after the intramuscular injection of anthiomaline in dose of 0.5 milligrams of Sb per kilogram of body weight. Levels are based on 6 patients (Otto, Maren, & Brown<sup>11</sup>).

hours in the plasma of 4 of the 6 patients. The principal variation from the average is seen in the higher levels after one hour in patients R.O. and B.G. Meanwhile, the cell concentration continued to rise or remain near peak levels in most patients through the 3-hour period, and, while it dropped rapidly thereafter, antimony was still conspicuously present at 24 hours in five of the six cases. Thus, the antimony cell-plasma ratio tends to be higher later in the 24-hour period than it is immediately after injection.

Otto, Maren, and Brown<sup>11</sup> noted that antimony levels in the blood cells were at their highest in the 1-2 hour period after the initial administration of anthiomaline, and, therefore, a series of blood samples was taken from two patients one hour after the administration of anthiomaline throughout the 21-day course of treatment. The average values for the two patients show a gradual increase in the antimony content of the plasma from 0.17 and 0.15 microgram of Sb per gram after the first and second dose to 0.36 and 0.62 micrograms per gram after the fifteenth and twenty-first doses.

On the other hand, no such trend was clearly shown with reference to the cells.

The urinary excretion of anthiomaline has been found by Brown and Spremuli to follow a similar pattern in man and in dogs. When the drug was injected intramuscularly, from 13 to 19 per cent of the antimony was excreted in the first 24 hours and 24 per cent within 48 hours (FIGURE 2).

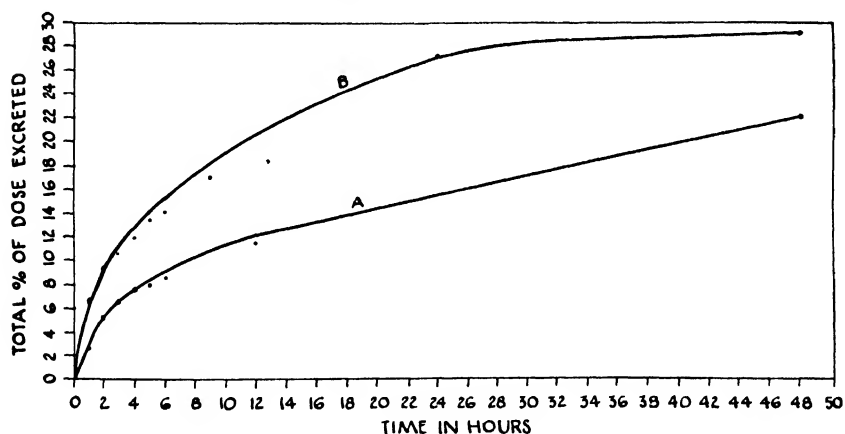


SUBJECT 1  
DOSE: 0.46 MG. SB PER KILO  
SB AS ANTHIOMALINE  
GIVEN INTRAMUSCULARLY

SUBJECT 2  
DOSE: 0.57 MG. SB PER KILO  
SB AS ANTHIOMALINE  
GIVEN INTRAMUSCULARLY

FIGURE 2. Urinary excretion of antimony by man.

As would be expected, however, the urinary excretion of anthiomaline was somewhat more rapid after intravenous injection than after intramuscular injection (FIGURE 3). Maren, Otto, and Brown<sup>11</sup> found 6-15 per cent of the anthiomaline to be excreted in the urine in the first 24 hours.



DOG A  
0.63 MG. SB PER KILO  
GIVEN INTRAMUSCULARLY

DOG B  
0.65 MG. SB PER KILO  
GIVEN INTRAMUSCULARLY

FIGURE 3. Excretion of antimony given as anthiomaline.

Recently, Brady *et al.*<sup>12</sup> have made use of radioactive antimony to determine the antimony concentrations in various organs following intravenous injection of tartar emetic. Analysis of dogs tissues 36 hours after injection of 0.8 mg. antimony as tartar emetic gave the following results (TABLE 8) :

TABLE 8

Micrograms of antimony per gram of tissue			
Liver	10.7	Retroperitoneal lymph node	0.35
Thyroid	3.8	Lung	0.34
<i>D. immitis</i>	1.48	Diaphragm	0.33
Kidney cortex	0.98	Kidney medulla	0.32
Pancreas	0.64	Thigh muscle	0.32
Jejunum	0.54	Stomach	0.24
Spleen	0.54	Left auricle	0.23
Left ventricle	0.53	Right auricle	0.22
Inguinal lymph node	0.48	Dermis	0.14
Right ventricle	0.39	Blood	0.12
Adrenal	0.37	Cortex of femur	0.03
Epidermis	0.37		

Of special interest was the relatively high concentration of antimony in the adult *D. immitis*, which suggests a specific affinity of the drug for the tissues of the worm. The concentration of antimony in the lymph nodes which are closely associated with *W. bancrofti* of man was low—only 0.48 micrograms per gram of tissue. Cowie *et al.*<sup>13</sup> made similar studies of antimony concentrations following 12 injections of sodium, antimonyl xylital. They found that the antimony in the whole blood rose from 0.02 micrograms per cc. wet blood on the second day to 0.15 micrograms on the last day of treatment. The thyroid gland had the greatest concentration of antimony, 18.72 micrograms per gram of tissue, followed by the liver, 13.75 micrograms; parathyroid glands 4.49; and *D. immitis* adults with 3.28.

**The Use of Anthiomaline in Human Filariasis.** The use of anthiomaline against human filariasis began with the work of de Choisey,<sup>14</sup> who treated a patient with clinical manifestations of a *Loa loa* infection with anthiomaline and reported improvement in his condition. Apparently, no microfilaria counts were made on the patient's blood during or after treatment. A total of 35 cc. of anthiomaline was given intramuscularly to this patient over a period of 23 days. Poynton<sup>15</sup> injected anthiomaline directly into enlarged lymph glands of patients harboring *Wuchereria bancrofti* or *W. malayi*. The attacks of fever were reported reduced in number and intensity in these patients. Chopra and Rao<sup>16</sup> treated seven filariasis patients with anthiomaline. They gave two intramuscular injections weekly using up to a total of 30 cc. per patient. Only a temporary slight reduction in microfilariae was noted. Hawking<sup>17</sup> likewise failed to secure a permanent microfilaria reduction in 10 *W. bancrofti* infected patients with anthiomaline given intramuscularly. The total dosage employed by

him varied from 7 to 25 cc., given over a period of from 12 to 49 days.

In spite of the failure of anthiomaline in the above trials, our experience with dogs suggested that, given in sufficient doses, it might be effective in man. Although the adult *Dirofilaria* lives free in the right cavity of the dog's heart and *Wuchereria bancrofti* of man lives in the lymphatic system, it was considered worth while to try anthiomaline in human infections. Our dog studies suggested that somewhat larger total doses than had been tried by other workers might be needed to be effective in human infections. It is quite conceivable, however, that a drug which reaches sufficient concentrations in dog's blood to kill worms therein may not reach sufficiently high concentrations in the human lymph system to be lethal to worms harbored there.

Anthiomaline has also been used with some success in the treatment of lymphogranuloma venereum,<sup>18, 19</sup> trypanosomiasis,<sup>20, 21</sup> schistosomiasis,<sup>22, 23</sup> leishmaniasis,<sup>24, 25</sup> and the tolerated dosage has been approximately ascertained and toxicity for man studied.\*

**METHODS.** All of the patients reported in our studies (Brown,<sup>26</sup> Brown and Thetford<sup>27</sup>) were natives of St. Croix, Virgin Islands. They were in the hospital for some cause other than filariasis or were found to be microfilaria-positive through a blood survey. The only physical evidence of infection in most instances was a general glandular enlargement especially of the inguinal and femoral lymphatic nodes. Some of the patients gave a past history of lymphangitis and several of them had hydroceles. None of the patients had what could be considered as elephantiasis.

Physical examinations were made before treatment of the patients, some of whom remained in the hospital during treatment while others were treated in an out-patient clinic without interruption to their usual daily work and activity.

All injections of lithium antimony thiomalate were given into the gluteal muscles, alternating sides daily unless the patient complained of soreness on one side and asked for repeated injections into the other side. Usually, a single daily injection was given on consecutive days unless prevented by poor cooperation of the patient or, rarely, by toxic manifestations due to the drug. The adult dose of 3 cc. (180 mg. anthiomaline, 30 mg. of antimony) was routinely used, except for 2 patients who were given 3.5 cc., with adjusted smaller doses for children. On the first day of treatment, a reduced dose was usually given to ascertain sensitivity of the patients to the drug. In order to keep the drug level as constant as possible with a single daily dose, the injections were given at regular twenty-four hour intervals.

The results of the therapy were measured by microfilaria counts on 0.1 cc. of blood after the method of Brown and Sheldon.<sup>2</sup> Since the microfilariae exhibit a nocturnal periodicity, all routine specimens of blood were drawn from the cubital vein between 10 and 10:30 p.m. In a number of

\* For a review of the literature on anthiomaline, see "A Summary of Current Literature on Anthiomaline," Office of Medical Information, National Research Council, 1943.



instances, blood was also secured at 10 a.m. to check the effect on the diurnally appearing microfilariae.

Microfilaria counts on the blood of control persons not treated were made and remained relatively constant during the time that the treated patients were under observation.

**RESULTS.** The results of treatment are based entirely on microfilaria counts. In assaying the results, it is evident that a drug used in the treatment of filariasis may kill the microfilariae in the blood stream without being fatal to the adult worms in the lymphatic system. In such an event, microfilariae would disappear temporarily from the blood stream but again appear in the blood after treatment is discontinued, due to reproduction by the adult worms. It is necessary, therefore, to examine the blood for months after treatment has ceased to ascertain whether or not the adult worms, as well as the microfilariae, are killed. One must also remember that the adult females may be sterilized by the drug rather than killed by it. Microfilaria counts have now been made on the patients treated with anthiomaline for as long as 24 months after the completion of treatment.

Anthiomaline from two sources was utilized: that used in the first group of patients treated was of French manufacture; later the drug manufac-

TABLE 9  
EFFECT OF INTRAMUSCULAR INJECTIONS OF FRENCH\* ANTHIOMALINE ON  
*Wuchereria bancrofti* INFECTIONS

Age	Treatment period (days)	Microfilariae in 1.0 ml. of night blood			Percentage reduction
		Before treatment	After treatment	12-24 months after treatment	
28	26	159	1	0	100.0**
11	28	1,666	3	0	100.0
31	17	234	58	0	100.0
28	26	22	5	0	100.0
11	18	756	18	1	99.9
16	18	1,100	25	22	98.0
70	17	208	21	6†	97.1
29	25	498	92	17	96.6
17	19	2,324	14	103	95.7
13	10	263	233	16	93.9
20	21	72	3	6	91.7
14	26	513	73	57	88.9
16	16	236	68	33††	86.0
23	13	86	109	19	77.1
6	17	368		93	74.7
21	7	129	8	37	71.4
19	20	12	42	6	50.0
28	28	23	9	18	21.7

\* Manufactured by Société Parisienne d'Expansion Chimique Spéciale, France.

\*\* Negative 48 months after treatment.

† Four-five months after treatment.

†† Seven months after treatment.

tured by Merck and Company was used. The therapeutic results on patients treated with drugs from these two sources have been kept separate to ascertain any possible difference in their toxicity or activity.

The effect of therapy lasting from 7 to 28 days using the French drug are given in TABLE 9. Sixty-one per cent of the patients exhibited a 90 per cent or greater drop in microfilaria count, while four of the 18 persons became microfilaria-negative by the Knott technique. The remaining 39 per cent of the group experienced microfilaria reductions ranging from 88.9 to 21.7 per cent. The total drop in microfilaria count for the whole group was 95.1 per cent. In general, there was a marked drop in the microfilaria count during treatment and a slow decrease for the following 12 to 24 months. Patient No. 5, for example, exhibited a drop in microfilaria count from 1,666 per 0.1 cc. of blood down to 3 by the completion of 28 days treatment (FIGURE 4). Microfilaria counts of from 3 to 12 per

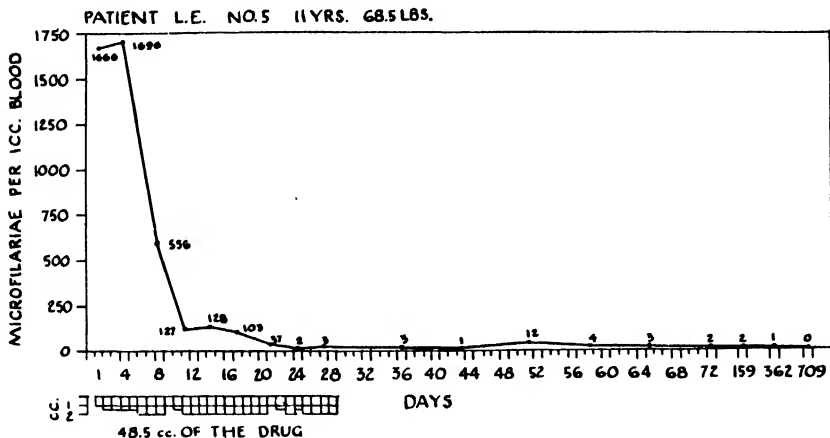


FIGURE 4. Effect of intramuscular injections of anthiomaline upon the microfilaria count of *Wuchereria bancrofti*.

0.1 cc. were noted over the following 12 post-treatment months. In the 24th month, the count fell to zero. The microfilaria count of patient No. 22 fell from 129 to 8 by the completion of treatment, but 12 and 24 months later the count was 29 and 37, respectively. Such a result might be explained by the drug killing most of the microfilariae in the blood stream but leaving a few adults alive, which gradually resulted in the increase in microfilariae. It is possible that reinfection following treatment might also be responsible for the late gradual increase in microfilariae.

The treatments with the American anthiomaline were designed to ascertain how many injections are necessary to achieve good therapeutic results. Groups of patients were therefore treated daily for 8, 15 or 22 days, with the results shown in TABLE 10. It will be noted that, in general, the longer the period of treatment, the better were the results in terms of microfilaria reduction. Although there were two patients with 100 per cent

TABLE 10

EFFECT OF INTRAMUSCULAR INJECTIONS OF AMERICAN\* ANTHIOMALINE ON  
*Wuchereria bancrofti* INFECTIONS

Age	Treatment period (days)	Microfilariae in 1.0 ml. of night blood			Percentage reduction
		Before treatment	After treatment	10-18 months after treatment	
23	22	133	53	2	96.2
21	22	654	20	33	95.0
32	22	177	25	15	91.5
20	22	58	7	—	88.0
47	22	170	20	42	75.3
25	22	104	72	65	37.5
17	15	1,108	34	0	100.0
20	15	49	12	0	100.0
30	15	44	21	16	63.7
24	15	171	88	70	59.1
33	15	1	0	1	0
8	8	31	18	0	100.0
70	10	62	1	7**	87.7
32	8	175	163	72	58.9
24	8	158	174	81	48.8
22	8	264	72	190	28.1
49	8	217	441	159†	27.8

\* Manufactured by Merck & Co., Rahway, N. J.

\*\* Two-four months after treatment.

† Five-eight months after treatment.

microfilaria reduction in the 15-day treatment series, one in the 8-day treatment series, and none in the 22-day treatment group, the latter group exhibited a more uniformly successful reduction in microfilaria count. The success attending the short series of injections suggests that, in some patients, the adult filariids are so located as to be more readily available to the drug.

The wide variations in the results of treatment may be explained in two ways. In some instances, the adult worms may have stimulated extensive fibrosis about themselves, making access of the drug to them difficult or impossible. The second explanation for the failure of therapy is indicated by the finding of Otto, Maren, and Brown<sup>11</sup> that plasma levels of antimony may vary in individuals more than 100 per cent even though the dosage on a per kilogram of body weight is the same. The first hypothesis does not permit study, but with the development by Maren<sup>10</sup> of a method for determining antimony blood levels, that later hypothesis may be studied. To test the second hypothesis, two patients (Nos. 23 and 26) in whom microfilaria reductions of 100 and 96.6 per cent, respectively, were obtained by treatment, were compared to patient No. 9 whose treatment resulted in a questionable, if any, reduction in microfilariae. Twelve to 24 months after their original anthiomaline therapy, all three patients were each given comparable per kilogram doses of the drug (0.5 mg. antimony per kilogram weight) and the blood plasma and red blood cell antimony concentration

followed at intervals. The results are shown in FIGURES 5 and 6. Both the plasma and red blood cell antimony levels of patient No. 9, who was unsuccessfully treated, were continuously as high or higher than those of patients No. 23 and 26 who were successfully treated. If these antimony

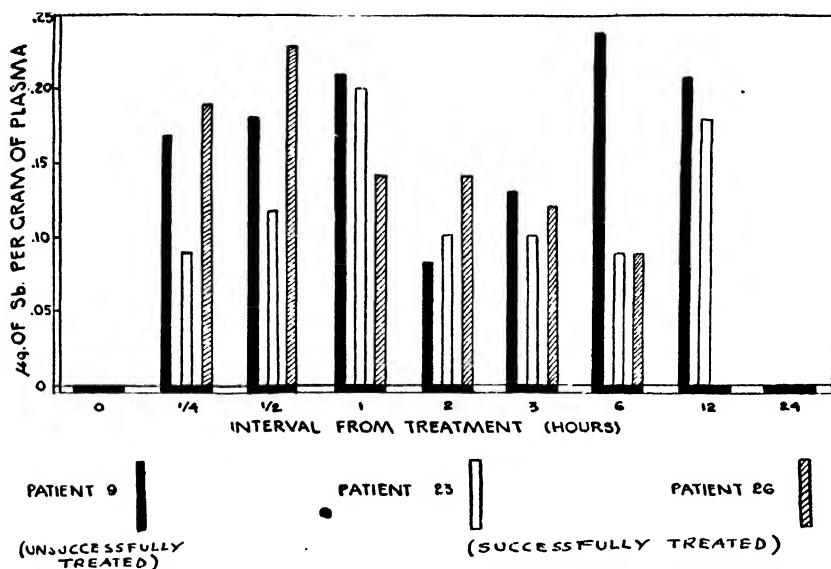


FIGURE 5. Antimony concentration in plasma in patients receiving 0.5 mg. of antimony as lithium Sb thiomalate per kilogram of body weight.

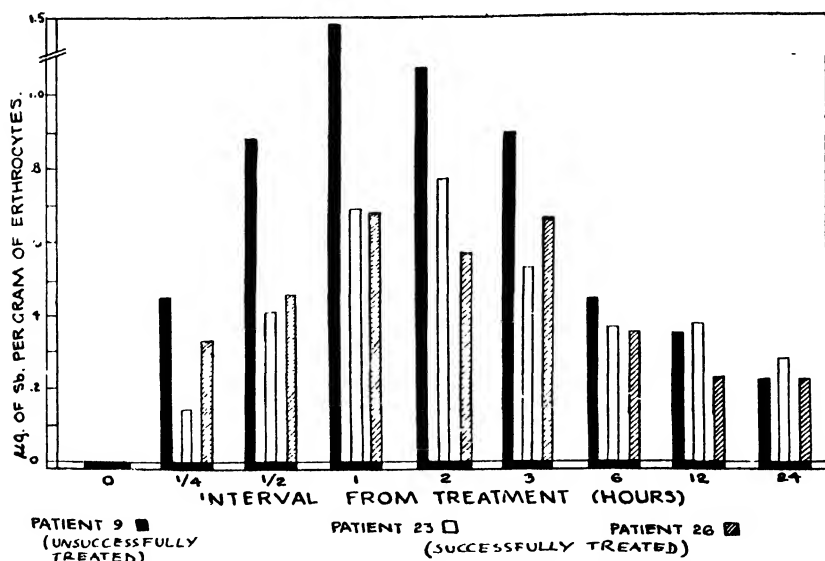


FIGURE 6. Antimony concentration in erythrocytes in patients receiving 0.5 mg. of antimony as lithium antimony thiomalate per kilogram of body weight.

blood levels are characteristic of the levels attained by the patients during their previous treatment, it does not appear that they could explain the differences in the success of the treatment. These studies suggest that the adult worms of patient No. 9 were inaccessible to the drug or were unusually resistant.

It has been suggested, by O'Connor,<sup>28</sup> that the pathological changes in filaria-infected persons result from the host reaction to the dead adult worms. The results of any therapy that has as its purpose the killing of the adult worms and microfilariae should, therefore, be followed for a considerable period to ascertain the patient's reaction to the death of both the adult worms (if this occurs) and the microfilariae, if their decrease in number is due to lethal action of the drug rather than to a normal rate of death which is not followed by normal reproduction by adults. If it is true that dead worms cause the pathological changes, then the large numbers of microfilariae (23,240 per cc. in patient No. 17) and adult worms should, in time, result in inflammatory reactions. On the other hand, tremendous numbers of microfilariae must die every day and be taken care of by the host in the average untreated case of filariasis, for if the length of life of a microfilaria is 60-70 days,<sup>29</sup> then 1/60 to 1/70 must die each day and be replaced. Patient No. 17, mentioned above, had an estimated 100,000,000 microfilariae in his blood stream, hence approximately 1½ million would die each day. This continued destruction of microfilariae by the host may partially desensitize him to the foreign protein of the worms.

Thirty patients treated with anthiomaline have now been followed clinically for 24-48 months, and no evidence of inflammation, lymph stasis or incipient elephantiasis has been detected. Our evidence, to date, indicates that the death of the adult worms and their millions of microfilariae offspring has not produced, in the host, a reaction that is clinically recognizable.

The treatment of filariasis after the patient becomes microfilaria-positive may be analogous to the treatment of syphilis after the patient exhibits a positive serology. In syphilis, it is much more satisfactory to start treatment earlier on the basis of a positive dark field examination. Likewise, in filariasis, during the year or two between infection and becoming microfilaria-positive, the host reaction about the worm and growth of the worm may make it much more difficult to eliminate the infection. It might be highly advantageous, therefore, to treat a filaria infection as early as a diagnosis can be made. Clinical and serological diagnosis of filariasis sometimes may be made within 3 to 5 months after infection (*i.e.*, one to two years before microfilariae appear in the blood), and it is suggested that a cure of these early cases may be successful inasmuch as the worm is immature, smaller, and presumably not as robust as the full grown adult. Likewise, tissue reactions about the young worm may be at a minimum and permit access of the drug to them in higher concentrations. A group of these early clinical cases that were microfilaria-negative were treated with anthiomaline. As they were service men during the war period, the

follow-up was difficult and incomplete. Data secured from this group suggest that anthiomaline had little, if any, effect on the clinical course of the disease.

**Toxicity of Anthiomaline to Patients.** **VOMITING.** The most frequent toxic reaction, experienced by 30 per cent of the patients, was vomiting accompanied by epigastric pain. Vomiting occurred only after repeated injections totaling from 0.780 to 1.89 grams of anthiomaline. Vomiting occurred most frequently 2 to 3 hours after the injection, although it was sometimes delayed 10 to 12 hours. In several instances, the vomiting ceased although treatment was continued; usually, however, once vomiting began, it continued as long as the full dose of the drug was continued. Pain in the epigastric region was complained of by the patients both before and after vomiting, and pain on pressure was noted in this area. To stop the vomiting, the dose of the drug was reduced or treatment omitted entirely for one day. Ashkar<sup>22</sup> and others have likewise reported vomiting after a total of 1.20 g. or more had been reached. Gobert<sup>23</sup> suggests a 2-hour rest after antimony treatment to reduce vomiting. In the series reported here, however, vomiting occurred among those patients who were hospitalized as well as those treated in the out-patient department.

**LOCAL REACTION AT THE SITE OF INJECTION.** Gluteal reaction at the site of injection of anthiomaline varied greatly from patient to patient. The majority of them experienced no inconvenience. Occasionally, a patient would complain of soreness and ask for the next several injections to be given in the opposite side. One female taking her injections in the out-patient department stopped treatment after 7 injections, totaling 0.9 grams of the drug, complaining of sore buttocks. Examination failed to reveal evidence of inflammation sufficient to warrant discontinuing treatment. Sites of former drug injections frequently could be palpated as firm intramuscular painless masses. There was no evidence of severe inflammation or abscess formation, and, in general, the reaction at the site of injection was of little moment.

Grenierboley<sup>30</sup> and Moulinard<sup>31</sup> stress the local and general tolerance to intramuscular injections of anthiomaline, while Chopra and Rao<sup>16</sup> report that anthiomaline caused severe reaction with pain and swelling at the site of injection. The latter were using doses of up to 5.0 cc., which may explain the severe reactions. The maximum dose used in the series reported here was 3.5 cc.

**FEVER.** Four patients experienced fever near the end of their series of treatment. One patient's temperature rose suddenly to 103° F. after 2.61 grams of anthiomaline and fell to normal over a period of 3 days when the drug was discontinued. Another patient ran a low grade fever of 99.5 to 100.2 during the last 7 days of treatment. Sezary and Bolgert<sup>32</sup> and others have likewise noted fever, usually during the last days of a series of injections.

**ARTHRITIS.** Although other workers<sup>32</sup> report localized pains of arthritic character as the most frequent toxic reaction of anthiomaline therapy, it was experienced by only two of the patients in this series. After 1.71 grams of drug, one patient (No. 25), weighing 125 pounds, noticed pain in his right shoulder area. Treatment was continued, and on the following day pain was noted also in his left shoulder. As the treatment continued, the pain extended down the flexor surfaces of his arms to his elbows and became so severe that he moved his arms only with great pain. Treatment was suspended for a day with marked improvement of his arms and shoulder. The pain seemed to be localized in the joints, tendinous insertions, and even in the muscles. The areas of the joints were very sensitive to pressure. There was no alteration in reflexes or edema.

**URINE.** Intravenous phenolsulfonphthalein tests were made on the patients to ascertain kidney function before treatment. Only one patient (No. 25) exhibited an excretion rate below the so-called normal. This patient's 15-minute excretion was 8 per cent and one-hour excretion 30 per cent. The patient tolerated the early portion of the treatment very well, but late in the treatment series he experienced one of the two arthritic disturbances encountered, and he also vomited.

Examinations of the various patients' urine (sugar, albumin, reaction casts, and blood cells) were made at intervals during treatment, and there was no evidence of renal damage. One patient, No. 5, had a trace of albumin in his urine before starting treatment. A trace of albumin was found in his urine on the 10th day of treatment after a total of 24.5 cc. of anthiomaline had been administered. His urine was free from albumin thereafter. Patient No. 23 showed a trace of albumin in his urine after 37.5 cc. of anthiomaline, but another specimen examined 6 hours later was albumin-free. On the 20th day of treatment, after 58.5 cc. of the drug, he again had a trace of albumin in his urine.

**RASHES.** Six of 12 patients who were especially carefully followed experienced transitory itching rashes after a number of injections of the drug. In three instances, the rash was on the lateral aspects of the fingers and thumbs and consisted of small raised papules 1 to 2 mm. in diameter. One patient had a similar rash on his face, especially his forehead, as well as his hands. Another patient had a punctate red rash on chest and flexor surfaces of his arms, and one patient had a similar rash on his chest and abdomen only. The rashes were not severe, and usually remained static or disappeared even though treatment was continued. There is some question as to the exact etiology of the rashes and their relation to the anthiomaline treatment.

**BLOOD.** Sezary and Bolger<sup>32</sup> believe that prolonged administration of anthiomaline seems to cause a certain degree of anemia. On the other hand, Ashkar<sup>22</sup> observed no change in the blood picture "except for the eosinophilia that usually occurs with the other antimony compounds."

Brooks<sup>4</sup> using dogs, followed the blood picture after an intravenous injection of 4.0 cc. of anthiomaline with the following results:

"In both dogs used for this study there was a decrease of more than 50% in the total white blood cell count within 15 minutes after the drug was administered. Following this, there was a rapid rise to 17,000 in one dog and 19,000 in the other. This maximum degree of leukocytosis was reached in approximately 12 hours in both cases. Over a period of 96 hours, the total counts gradually decreased again to what was considered normal for these individual dogs. The red blood cell counts showed a brief rise following the injection of anthiomaline, after which there was a gradual decrease up to 20 hours. By the end of 96 hours, the counts were again normal."

Launoy and Lagodsky,<sup>33</sup> using anthiomaline in rabbits intravenously, concluded that 4-5 injections over 24-35 days produced only a slight transient anemia.

The blood of four patients in the series reported here was examined before treatment and 4 weeks and 4-5 months after treatment (TABLE 11).

TABLE 11  
BLOOD PICTURE OF ANTHIOMALINE TREATED PATIENTS BEFORE AND AFTER TREATMENT

Pt.	Before treatment			4 wks. after treatment			4-5 mos. after treatment		
	RBC	Hb %	WBC	RBC	Hb %	WBC	RBC	Hb %	WBC
4	2,880,000	62	9000	2,900,000	65	8900	3,400,000	70	6600
5	3,500,000	76	7600	3,650,000	70	8400	3,630,000	72	9200
18	3,480,000	68	7300	3,000,000	68	8800	2,980,000	65	8400
21	3,410,000	70	6800	2,440,000	60	6200	3,420,000	70	7000

The red cell count of patient No. 18 was approximately 500,000 cells lower 4 weeks and 4 months after treatment, although her hemoglobin remained unchanged. Patient No 21 had a drop of approximately 1,000,000 red cells 4 weeks after treatment but returned to pre-treatment level by 4 months after treatment. The variation in white blood cell counts were probably all within normal limits.

**Discussion.** The results of treatment of *Wuchereria bancrofti* infected persons with anthiomaline strongly suggests that the drug is lethal not only to the microfilariae but to the adult worms as well, and that from 85 to 100 per cent of the worms are killed by one series of the drug. It is possible, of course, that the microfilariae circulating in the blood are more delicate than the adults and are more readily killed. Likewise, a higher concentration of the drug may reach the microfilariae in the blood stream than reaches the adults in the lymphatics. Experience indicates that a drug may kill the microfilariae and temporarily sterilize the adult female



so that the production of microfilariae is inhibited for a time. It is essential, therefore, that the patient's blood be repeatedly examined for microfilariae over a considerable period after the completion of the treatment. The patients treated with anthiomaline have been examined for microfilariae for 24 months after the completion of treatment to determine the effect of the drug on the adult worms. It is considered unlikely that the adult worms that were injured to the extent that they did not produce microfilariae after 24 months are still alive.

Treatment with lithium antimony thiomalate usually resulted in decided reduction in microfilaria counts after a total of 15 to 20 cc. of the drug. This suggests that the microfilariae may vary in susceptibility to lithium antimony thiomalate and that the resistant ones were killed only after a longer exposure to the drug. It may also mean that the adult worms differ in the rapidity with which they were killed owing either to individual variation in resistance or to their localization in the host. The initial sudden drop in microfilariae, then, may have been due to their elimination from the blood stream by the direct action of the drug. The adults, presumably more resistant to the effects of the drug and more slowly killed, continued to supply the blood stream with fewer and fewer microfilariae.

The continued persistence of microfilariae in the blood 12-24 months after treatment suggests that adult worms are still present, possibly protected in a fibrosed lymph gland from lethal concentrations of the drug. Our present knowledge indicates that microfilariae persist in the blood approximately ten weeks. It is doubtful, therefore, if the continued persistence of microfilariae could have been possible for 24 months without the presence of the adults.

Anthiomaline was the first drug that has been shown to reduce permanently the microfilariae counts of individuals harboring *Wuchereria bancrofti*. Although this drug represents a great advance in the therapy of filariasis, it is obvious that the necessity for repeated injections attended by a certain amount of toxicity limits its usefulness, and it should be considered only as a stepping-stone to the search for an oral non-toxic therapeutic agent.

The use of anthiomaline in canine filariasis, although promising, awaits further study to ascertain a safe effective therapeutic regime. Additional studies must be made on the ultimate fate of the adult worms following treatment. Slow death of the adult *Dirofilaria immitis* would be advantageous as the risk of embolism to the host would be minimized, but if the worms are only sterilized therapy has been a failure except for the prevention of transmission to other dogs.

## SUMMARY

(1) Anthiomaline given intramuscularly to patients infected with *Wuchereria bancrofti* produces a rapid diminution in the number of circulating microfilariae which persists as long as two years.

(2) In one series of 18 patients treated for 7-28 days, 61 per cent experienced a microfilaria reduction of 90 per cent or more, and four of the group became microfilaria-negative. Treatment failed to reduce significantly the microfilaria counts of a number of patients.

(3) The therapeutic effectiveness of the French manufactured and American product (Merck) appear to be approximately equal. There appears to be a slight advantage of the American product in terms of decreased toxicity, which may be due to its more recent manufacture.

(4) The antimony blood plasma levels and erythrocyte levels in patients successfully treated did not differ appreciably from those of a patient unsuccessfully treated.

(5) No evidence of inflammation or incipient elephantiasis due to a reaction of the host to the death of microfilariae or adult worms has been detected in the patients over a period of two years.

(6) The toxic manifestations, due to anthiomaline, of vomiting, joint pain, slight fever, and rash are not considered sufficient to preclude its continued trial in filariasis.

(7) The microfilaria counts of dogs harboring *Dirofilaria immitis* were markedly reduced by anthiomaline therapy, but additional studies are needed to ascertain the exact fate of the adult worms in the heart.

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# TREATMENT OF FILARIASIS WITH NEOSTIBOSAN AND SOME OTHER COMPOUNDS

By JAMES T. CULBERTSON\*

Department of Bacteriology and Parasitology, School of Medicine,  
University of Arkansas, Little Rock, Arkansas†

Although filarial infection due to *Wuchereria bancrofti* has long been recognized as a serious tropical disease, no convincing data purporting to show successful chemotherapy of this affliction had appeared until a little more than three years ago.<sup>1</sup> At about the same time in 1944, two papers were published which indicated that this disease might be subject to chemotherapeutics. One of these papers, by Brown,<sup>2</sup> came from a study conducted in the Virgin Islands on human filarial subjects treated with the trivalent antimony compound, anthiomaline. During the succeeding years, Dr. Brown has been able to follow his original subjects as well as some additional patients, with continuing favorable results.<sup>3</sup> The other paper, by Culbertson and Rose,<sup>4</sup> showed that a natural filarial infection (*Litomosoides carinii*) of cotton rats could be eliminated when either of two pentavalent antimony compounds (neostibosan or neostam) was administered intensively to these animals.

Perhaps the most significant fact revealed by the work in the cotton rats was that, in this rodent infection, the drugs had their first and greatest activity on the adult phase of the infecting parasite and showed very little action against the embryonic stage (microfilaria) which occurred in the peripheral blood. For example, when animals were autopsied after a few days of treatment, all the adult parasites were frequently found dead in the pleural space—their natural habitat—and enveloped, more or less as foreign bodies, in fibrinous exudate. Surprisingly enough, at that time, the number of circulating microfilariae was essentially the same as before treatment had been initiated. If, however, the animals were allowed to live on after treatment, the number of circulating embryos gradually diminished until, in a few weeks or months, none whatever could be found. It finally became clear that the embryos disappeared from the blood not because of direct antagonistic effects of the drugs on the embryos, but because the drugs killed the parent worms. Since no additional embryos were produced once the adult worms were dead, the blood of treated rats finally became negative when those embryos which were present at the time of treatment were disposed of by whatever antagonistic forces the rat could bring against them. With these facts in mind and with the thought that the same drugs which were active in the rat might also be active against the adult base of the human filaria, a fairly comprehensive

\* Present address: Division of Research Grants and Fellowships, National Institute of Health, Bethesda, Maryland.

† The work reported in this paper was performed in collaboration with Dr. Harry M. Rose, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, N. Y., and Dr. José Oliver-Gonzalez, Department of Medical Zoology, School of Tropical Medicine, San Juan, Puerto Rico.

experimental study of the chemotherapy of human filariasis was inaugurated in Puerto Rico, with the generous cooperation of the Staff of the School of Tropical Medicine in San Juan.\*

## MATERIALS AND METHODS

**The Patients.** Altogether, 129 subjects infected with *Wuchereria bancrofti* were included in this study. All were native Puerto Ricans (except 1, from Martinique) whose nocturnal blood harbored microfilariae. The patients were in good general health, only 3 showing symptoms of filarial infection (2 with swollen legs, 1 with chyluria). Many of the patients (those below 18 years of age) were students in children's homes near San Juan who had been discovered to harbor the infection through a survey of all inmates of these institutions. Most other patients were adult males who had first learned of their infection when rejected for service by the military because of the presence of microfilariae in their night blood. Fifteen of the patients were kept untreated as controls of the infection and of the effects of treatment.

**The Drugs.** The following drugs were employed in the study: four pentavalent antimonials (neostibosan, neostam, urea stibamine, and stibanose); three trivalent antimonials (fuadin, anthiomaline, and tartar emetic), and one arsenical (melarsen oxide). All of these preparations were given intravenously except stibanose, fuadin, and anthiomaline, which were administered intramuscularly. Melarsen oxide was given orally to a few patients and intravenously to others.

Most of these drugs had been tried with some success by Dr. Rose and myself in the cotton rat filariasis before we went to Puerto Rico. Others had been recommended to our use by Dr. L. R. Farquhar, of the National Research Council, from trials in the cotton rat infection reported to her by Drs. R. N. Bieter (University of Minnesota), G. F. Otto (Johns Hopkins University), H. J. Robinson (Merck and Company), and A. D. Welch (Western Reserve University).

**Estimation of Level of Infection.** The level of infection in patients was estimated before treatment, at the end of treatment, and at intervals after treatment ceased, by counting all the microfilariae in 60 cmm. of nocturnal blood, this blood always being drawn at precisely the same hour for every observation in a given patient. Dehemoglobinized blood films were fixed in alcohol ether, stained with Ehrlich's hematoxylin, and destained in acid alcohol. Parasites were counted under X100 magnification of the microscope. From selected patients, including most of those whose blood was negative by the 60 cmm. samples, 10 cc. of blood were

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obtained, these being taken at or soon after 10:00 p.m. The large samples of blood were treated with 2 per cent saponin in physiological salt solution and centrifuged, and the sediment which remained was washed several times in salt solution. Finally, the total residue was examined under X100 magnification of the microscope.

## PROCEDURE AND RESULTS WITH SPECIFIC DRUGS

Of the 114 patients treated, 35 were given neostibosan, 11 neostam, 6 urea stibamine, 5 stibanose, 15 fuadin, 20 anthiomaline, 4 tartar emetic, and 18 melarsen oxide. The results obtained with these different drugs follow. We shall present in considerable detail the results obtained by administering neostibosan, since the group given this drug was the largest of all and showed the most promising results. A number of preliminary reports have already appeared from this work.<sup>5, 6, 7</sup>

NEOSTIBOSAN (Winthrop Chemical Company). The subjects given neostibosan can conveniently be divided into 3 groups: a first group of 20 who received comparatively mild treatment over a prolonged period (generally 34 to 54 days), a second group of 5 given intensive therapy for a shorter interval (two weeks), and a third group of 10 who were given both the light course and, 9 months later, the intensive course of therapy. These patients have been observed during the months since treatment ceased, the period of observation being as long as 3 years for some and at least 26 months in all others (see TABLES 1, 2, and 3).

NEOSTIBOSAN (Winthrop Chemical Company). The subjects given neostibosan can conveniently be divided into 3 groups: a first group of 20 who received comparatively mild treatment over a prolonged period (generally 34 to 54 days), a second group of 5 given intensive therapy for a shorter interval (two weeks), and a third group of 10 who were given both the light course and, 9 months later, the intensive course of therapy. These patients have been observed during the months since treatment ceased, the period of observation being as long as 3 years for some and at least 26 months in all others (see TABLES 1, 2, and 3).

The tables of data on these subjects show that, of the 20 patients in the first group, 14 became negative for microfilariae and have remained so for periods up to three full years. Of the 6 others in this group, 3 when last seen had sharply reduced numbers of microfilariae in their circulating blood compared with the number present before treatment began. The 3 remaining persons all had become negative for parasites during part of the period of observation, but at the last examination 2 of them had shown enough parasites to indicate either reinfection or else relapse of the old infection.

Of the 5 patients given the short intensive course of therapy, 3 lost all microfilariae during the 27 months they have been followed, and the 2 others lost 95 per cent of their parasites. Of the third group of 10 patients, 9 lost all microfilariae within 26 months after the second course

TABLE 1  
DATA ON TWENTY PATIENTS WITH FILARIASIS GIVEN ONE COMPARATIVELY LIGHT COURSE OF NEOSTIBOSAN

Case No.	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times					Microfilaria level, per cent of change over entire period of observation		
					Before treatment	At end of treatment	Months after end of treatment					
							6	12	18		24	36
1 LET	11	60	7.2	40	3	0	0	0	0	—100		
2 MR	8	50	4.6	25	9	0	0	0	0	—100		
3 VR	10	48	5.8	34	24	6	0	0	0	—100		
4 AEB	16	106	7.2	40	15	36	0	0	0	—100		
5 RT	14	100	7.2	40	41	36	0	0	—	—100		
6 CIR	13	102	7.2	40	216	204	6	0	0	—100		
7 EMD*	18	138	10.4	48	18	42	2	0	0	—100		
8 MSC*	26	112	10.5	48	150	120	10	0	0	—100		
9 GM*	15	114	8.1	40	33	15	10	0	—	—100		
10 CP*	21	125	7.6	49	15	61	1	0	0	—100		
11 CAR	15	93	6.8	40	231	207	21	1	0	—100		
12 JOA*	20	133	9.2	54	82	8	52	1	0	—100		
13 PB*	11	58	6.9	39	177	96	111	22	0	—100		
14 RRC*	26	168	9.2	54	24	6	17	—	0	—100		
15 JG*	13	79	8.1	39	255	204	14	0	1	—98		
16 OA*	21	146	7.2	38	36	12	1	1	0	—97		
17 JL*	13	76	7.5	40	297	294	111	18	—	—97		
18 DG*	12	73	7.5	40	21	51	0	0	1	—57		
19 RMA	11	68	1.3	9	57	35	0	9	7	—24		
20 JT*	17	134	8.1	40	27	42	0	0	0	+136		

\* Males.

TABLE 2

DATA ON TEN PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*)  
GIVEN TWO COURSES OF NEOSTIBOSAN

Case No.	Age	Sex	Weight lb.	First course of therapy					
				Drug given g.	Days of treat- ment	No. of microfilariae in 60 cmm. blood at designated times			
						Before treat- ment	At end of treat- ment	Month after end of treat- ment	
								6.0	9.0
21 MN	12	F	74	6.5	40	27	54	13	13
22 ME	16	F	102	6.9	40	136	126	49	65
23 HRR	11	M	56	7.3	34	18	9	15	16
24 DR	13	M	76	8.1	40	72	120	39	67
25 CF	8	F	52	6.4	33	123	93	109	65
26 CD	16	F	112	7.1	40	120	126	42	31
27 FG	13	M	71	8.1	40	630	624	217	284
28 JR	16	F	85	6.0	33	154	129	81	69
29 BM	14	F	91	7.1	40	78	87	56	75
30 IO	14	F	138	7.2	40	129	156	87	71

Case No. ( <i>cont'd.</i> )	Second course of therapy							Microfilaria level, per cent of change over entire period of observa- tion	
	Drug given g.	Days of treat- ment	No. of microfilariae in 60 cmm. blood at designated times			Months after end of second course			
			Before second course	At end of second course					
						8	14		26
21 MN	7.9	10	13	1	0	0	1	—100	
22 ME	12.5	14	65	11	0	0	0	—100	
23 HRR	5.9	9	16	1	0	0	0	—100	
24 DR	11.5	15	67	9	0	0	0	- 100	
25 CF	9.0	11	65	0	1	0	0	- 100	
26 CD	12.0	14	31	21	2	0	0	100	
27 FG	11.1	15	284	60	15	0	—	—100	
28 JR	10.0	14	69	39	25	2	0	—100	
29 BM	12.5	14	75	40	18	8	0	—100	
30 IO	12.0	14	71	57	4	1	—	—96	



TABLE 3  
DATA ON FIVE PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) GIVEN A SINGLE INTENSIVE COURSE OF NEOSTIBOSAN

Case No. *	Age	Weight lb.	Drug given g.	Days of treat- ment	Number of microfilariae in 60 cmm. blood at designated times					Microfilaria level, per cent of change over entire period of observation	
					Before treat- ment	At end of treat- ment	Month after end of treatment				
							3.0	9.0	15.0		27.0
31 EG	8	46	11.0	15	30	0	0	0	0	—100	
32 MM	14	78	9.5	14	93	12	3	0	0	—100	
33 JAA	21	134	15.5	13	79	26	9	0	0	—100	
34 VN	11	65	11.0	15	651	150	234	90	39	—95	
35 CM	36	162	12.3	12	194	144	24	82	60	—95	

\* All are males.

TABLE 4  
DATA ON ELEVEN PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH NEOSTAM

Case No.*	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times					Microfilaria level, per cent of change over entire period of observation
					Before treatment	At end of treatment	Months after end of treatment			
							6	13	25	
1 MMR	22	113	10.6	14	366	121	16	0	—	—100
2 FT	22	140	7.7	15	189	175	1	0	0	—100
3 LMS	26	112	9.8	14	96	34	25	0	0	—100
4 JMG	21	115	8.5	15	55	34	39	0	0	—100
5 AAR	19	137	11.0	14	35	8	17	1	0	—100
6 RNV	25	175	11.4	14	249	25	1	10	—	—91
7 ALB	23	107	10.6	14	62	14	0	0	10	—83
8 AF	25	146	2.1	5	33	—	31	8	8	—75
9 CBC	23	134	10.8	14	21	4	4	6	—	—71
10 AVG	21	121	10.1	14	290	49	100	116	—	—59
11 TMO	19	128	8.7	15	165	47	187	—	—	+12

of drug, and the remaining individual (who could be followed for only 14 months) lost 95 per cent of his original number of parasites.

Taken altogether, of the 35 neostibosan-treated patients, 27 were entirely negative for microfilariæ when last seen and all but 2 of the remaining persons showed a marked drop in the number of circulating parasites. It should be pointed out that the drug was generally well tolerated by patients, although some individuals showed nausea, vomiting, epigastric pain, fever, headache, or salivation at some time during the course of treatment.

NEOSTAM (Burroughs Wellcome and Company). Eleven patients were intensively treated with neostam over a period of 2 weeks. Twenty-five months after the end of treatment, 5 of the eleven patients had lost all microfilariæ and 4 others had lost 75 per cent or more of the microfilariæ originally present (see TABLE 4).

UREA STIBAMINE (E. R. Squibb and Sons; Brahmachari, India). Six patients were intensively treated with urea stibamine for approximately two weeks. The group was observed for periods up to 28 months after treatment ceased. When last seen, all 6 individuals in this group were free of microfilariæ (see TABLE 5).

STIBANOSE (Winthrop Chemical Company). Five patients were treated with stibanose. When last seen, after periods up to 24 months, one individual was negative for microfilariæ and two others had lost over half their circulating embryos. Of the remaining two patients, one had lost only 4 per cent of his parasites and the other had 114 per cent more parasites than before treatment (see TABLE 6).

FUADIN (Winthrop Chemical Company). Fifteen subjects were treated with fuadin. Of these, 5 were negative for microfilariæ 26 months later, and 5 others had lost over 90 per cent of their circulating embryos (see TABLE 7).

ANTHIOMALINE (Merck and Company). Twenty patients were intensively treated with anthiomaline. Six were negative for circulating parasites when seen 26 months later, and 7 others had lost over 90 per cent of their embryos (see TABLES 8 and 9).

TARTAR EMETIC (Abbott Laboratories). Only four subjects were given tartar emetic. After 25 months, one had lost all filarial parasites, and 2 others had lost over 90 per cent of the microfilariæ originally present (see TABLE 10).

MELARSEN OXIDE (Parke, Davis and Company). Eighteen patients were treated with melarsen oxide, 3 of these receiving the drug by mouth and 15 being injected intravenously with a solution of drug in propylene glycol. After 26 months, 8 of the group were negative for circulating embryos, and one other had lost over 90 per cent of his parasites (see TABLE 11).

TABLE 5  
DATA ON SIX PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH UREA STIBAMINE

Case No.*	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times				Microfilaria level, per cent of change over entire period of observation
					Before treatment	At end of treatment	Months after end of treatment		
							6.0	16	
1 RCA†	25	146	6.8	16	3	4	0	0	—100
2 JFL†	19	144	7.1	17	20	5	0	0	—100
3 ALV†	30	141	7.1	17	155	181	128	16	—100
4 JBM	19	120	4.2	14	12	0	0	—	—100
5 MFT	20	127	3.3	11	14	18	0	—	—100
6 HM	21	109	4.9	16	334	96	163	10	—100

\* All are males.

† Given E. R. Squibb & Sons product; others given Brahmachari product (India).

TABLE 6  
DATA ON FIVE PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH STIBANOSE

Case No.*	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times				Microfilaria level, per cent of change over entire period of observation
					Before treatment	At end of treatment	Months after end of treatment		
							5	12	
1 JV	7	49	13.8	11	32	33	0	0	—100
2 VB	15	106	14.5	13	45	26	18	8	—82
3 MR	12	96	15.2	13	486	576	481	601	214
4 AD	10	54	15.2	13	129	96	105	118	123
5 RRA	12	79	15.2	13	155	159	87	217	333
									+114

—100

—82

—55

—4

+114

TABLE 7  
DATA ON FIFTEEN PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH FUADIN

Case No.*	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times					Microfilaria level, per cent of change over entire period of observation
					Before treatment	At end of treatment	Months after end of treatment			
							8	14	26	
1 JJ 2 SVA	29 31	124 144	2.7 4.5	11 13	145 287	44 132	2 13	0 0	0 0	—100 —100
3 RRZ 4 PRD	21 26	120 169	3.3 3.8	13 11	181 133	44 10	31 38	5 14	0 0	—100 —100
5 MNR 6 EMC	18 27	142 118	5.2 4.0	13 11	112 64	51 54	33 —	0 1	— 0	—100 —100
7 LFCR 8 GMF	22 18	126 122	4.2 4.8	12 14	389 154	29 40	59 8	27 1	1 3 Y	—99 —98
9 BAC 10 JRC	21 19	140 123	3.5 1.6	14 6	273 298	33 123	95 16	1 —	4 —	—98 —94
11 ARH 12 GG	23 30	126 113	4.2 6.1	13 14	852 234	89 31	315 68	139 30	57 —	—93 —87
13 JRA 14 SSP	18 22	127 132	5.0 4.2	14 13	517 493	161 46	240 99	203 —	71 —	—84 —79
15 SRM	24	133	2.5	7	233	79	174	—	—	—25

\* All are males.

TABLE 8  
DATA ON TEN PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH ANTHIOMALINE SPECIA

Case No.	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times				Microfilaria level, per cent of change over entire period of observation	
					Before treatment	At end of treatment	Months after end of treatment			
							7	14		26
1 VG*	17	118	3.1	17	47	3	0	0	—100	
2 CAC*	18	106	2.3	16	33	4	—	0	—100	
3 FVB	22	139	3.0	17	433	199	0	0	—100	
4 PV	18	111	2.4	14	951	180	166	50	—99	
5 BCR	23	133	3.6	20	289	15	47	0	—94	
6 JLT	20	111	3.6	20	677	132	212	143	—91	
7 GSM	35	107	3.6	20	722	163	69	276	—67	
8 GG*	15	143	1.1	7	139	115	36	45	—51	
9 RRM	22	124	3.6	20	1094	134	489	158	—85	
10 DVG	22	124	3.0	18	22	10	17	—	—22	

\* Females.

TABLE 9  
DATA ON TEN PATIENTS WITH FILARIASIS (*Wuchereria bancrofti*) TREATED WITH ANTHIOMALINE MERCK

Case No.*	Age	Wt. lb.	Drug given g.	Days of treatment	Number of microfilariae in 60 cmm. blood from treated patients at designated times					Microfilaria level, per cent of change over entire period of observation
					Before treatment	At end of treatment	Months after end of treatment			
							6	13	25	
1 PPP	36	146	3.3	13	74	0	2	0	0	—100
2 EGC	20	125	4.2	13	99	5	1	0	0	—100
3 MADB	19	153	2.9	13	317	175	0	0	0	—100
4 FF	21	129	1.6	6	348	248	—	11	2	—99
5 PRR	20	126	2.7	9	324	104	3	5	11	—96
6 MASR	27	130	2.0	13	99	78	—	4	—	—95
7 SGC	32	106	2.7	13	163	53	61	44	12	—92
8 MNR	21	119	2.3	13	133	111	6	33	32	—77
9 HGC	19	126	1.9	11	365	376	132	165	90	—75
10 LRS	18	139	2.3	13	1036	867	—	—	—	—16

\* All are males.

**Observations in Control Subjects.** All of the 15 control subjects remained in good health, so far as their filarial infections were concerned, during the entire period of observation which, for some, was as long as 38 months. It was of considerable interest that at no time during this period did any of the control subjects have a negative nocturnal blood sample. At the end of the full period of observation, 3 of the control patients showed fewer microfilariae in their circulating blood than at the beginning, but, when last seen, 12 of the 15 controls had more embryos than when observations began (see TABLE 12).

The rise in parasite numbers seen in most of the control subjects can probably be explained through the comparative youth of most of these individuals (8 to 17 years of age). The infections in these persons, as in many of the treated subjects, may have been newly acquired. The gradually rising embryo counts may, then, reflect a gradual accumulation of embryos in the blood of these patients.

**Effect in Adult Worms.** In most patients, no effects were seen which could be interpreted as a deleterious action by drug upon the adult filarial worms. In a few adult male patients, however, one or two tender nodules, ranging up to 1 cm. in diameter, appeared in the scrotal sac late in the course of therapy or during the first 10 days after treatment ceased. One nodule was surgically removed by Dr. J. S. Colon, Urologist of University Hospital in San Juan, and sections of this nodule were prepared and studied by Dr. Enrique Koppisch, Pathologist of the School of Tropical Medicine. The sections revealed a filarial worm surrounded by an extensive inflammatory area suggestive of an Arthus reaction. From the condition of its ovarian nuclei, the worm was considered to be recently dead.

Naturally, the appearance of these nodules in the scrotal sac was of the greatest concern not only to the treated patients but to ourselves. In the cases of other patients besides the one already referred to from whom a nodule was excised, the reaction subsided in 2 weeks or so, the nodules gradually regressing in size thereafter to become tiny hard granules which usually persisted for several months. The reactions were seen in patients given either trivalent or pentavalent antimony, as well as in patients given the arsenical. It is not believed that any of the patients suffered significant or permanent impairment as the result of the formation of these nodules which we have assumed to represent parent worms killed by the administered drug.

**Blood Levels of Antimony.** It was of interest to know to what extent antimony accumulated in the blood of patients and how, if at all, this was related to the disappearance of microfilariae. Detailed studies along these lines were carried out and have been presented elsewhere. For present purposes, suffice it to say that substantially higher plasma levels were attained with pentavalent antimony preparations (up to 1.0 mg. % with neostibosan) than with trivalent antimonials. Unfortunately, however,

it was difficult to draw any conclusion at all as to a possible correlation between high antimony levels and the disappearance of microfilariae from blood. Indeed, many patients treated with antimonials which attained only trace levels in the plasma lost most of their microfilariae (in some cases all of them) during the course of therapy.<sup>8</sup>

**Discussion.** It will be helpful, for evaluating the data on all patients, to indicate the number of individuals treated with each drug who were apparently freed of filarial infection. The information is given in TABLE 13.

TABLE 13

Drug	Number of patients treated	Number of patients freed of infection	Months of observation
Neostibosan	35	27	26-36
Ncostam	11	5	25
Urea stibamine	6	6	28
Stibanose	5	1	24
Anthiomaline	20	6	26
Fuadin	15	6	26
Tartar emetic	4	1	25
Melarsen oxide	18	8	26

It appears from these data that, following the intensive application of any of several compounds of antimony or arsenic, filarial infection due to *Wuchereria bancrofti* can be eradicated from human subjects. None of the drugs used in the present study has proved to be an ideal preparation for use in man, for, with all of them, intensive administration by syringe over a considerable period of time was necessary in order completely to eradicate the infection. Furthermore, as has been indicated in earlier reports on this work,<sup>6</sup> severe if not dangerous reactions were frequently seen during treatment with any of these compounds. In fact, it is probably true that the hazard from the existing filarial infection was less in most subjects than that from toxic effects from the administered drugs. Practical therapy of filariasis, therefore, must await the discovery of a filaricidal drug which is safer to use than are those here employed and which exercises its effect in shorter time. It would be helpful, too, if this compound could be given orally.

## CONCLUSION

*Wuchereria bancrofti* can be eradicated from many patients by the intensive administration of any of several compounds of antimony or arsenic. Of the compounds thus far tried, the pentavalent antimonial, neostibosan, has shown promise in the treatment of this disease because it is well tolerated by patients and has a marked destructive effect on the parasites.



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# EXPERIMENTAL THERAPY OF ONCHOCERCIASIS WITH TRIVALENT ANTIMONIALS\*

By F. C. BARTTER, T. A. BURCH, D. B. COWIE, L. L. ASHBURN,  
AND F. J. BRADY

*Division of Tropical Diseases and Pathology Laboratory, National Institute of Health, Bethesda, Maryland, and Carnegie Institution of Washington, Washington, D. C.*

Until the last few years, the chemotherapy of human filarial infections has been notoriously unsuccessful in spite of the fact that *Dirofilaria immitis* infections of the dog have been known to be amenable to treatment with trivalent antimonials since the work of Itagaki and Makino<sup>1</sup> in 1927. However, Brown,<sup>2</sup> in 1944, using lithium antimony thiomalate (Anthio-maline), and Culbertson, Rose, and Oliver-Gonzalez,<sup>3</sup> in 1945, using pentavalent antimonials, reported the reduction or disappearance of microfilariae of *Wuchereria bancrofti* following treatment with these compounds.

Experimental chemotherapy of persons infected with *Wuchereria bancrofti* allows the quantitation of microfilariae in the blood but has the disadvantage that the effects of therapy upon adult parasites can be learned only by inference. In infections with *Onchocerca volvulus*, the contrary is true in that no technique has been devised that permits of more than a crude estimation of the numbers of microfilariae; however, the adult parasites may be recovered and examined to determine the effects of the drug.

With the filariae available for laboratory research, evidence indicates that antimonials have a differential activity upon microfilariae and adults dependent upon the species of parasite. In the case of *Dirofilaria* infections, the effect upon microfilariae is readily demonstrated. Furthermore, Ashburn *et al.*<sup>4</sup> have added a further criterion of therapeutic effect by showing that changes take place in the reproductive system of adult female worms from effectively treated animals, changes that are evident only on microscopic examination of multiple levels of the adult filaria.

Failure of chemotherapeutic agents in onchocerciasis has been attributed to failure of the drug to reach the parasites in adequate concentrations because of their location in connective tissues. In the case of antimonials, two methods of investigation of this hypothesis were available to us, namely, histological examination of adult worms and measurement of antimony uptake by adult worms after treatment with radioactive antimony.

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## EXPERIMENTAL PROCEDURE

Facilities for experimental chemotherapy of volunteers with onchocerciasis were provided for us through the courtesy of the Dirección General de Sanidad Pública de Guatemala. Twenty-five volunteers were brought from the endemic zone into Guatemala City.\* Pre-treatment examina-

TABLE 1  
DOSAGES USED TO TREAT PATIENTS WITH ONCHOCERCIASIS

Patient No.	No. doses	Mg. anti- mony/kg. body wt./day	Duration of treatment, days	Total Sb mg./kg.
		<i>Stibophen</i>		
1.	12	0.46	17	5.5
2.	25	0.46	24	11.4
3.	25	0.46	24	11.4
4.	25	0.46	24	11.4
5.	25	0.46	24	11.4
6.	25	0.46	24	11.4
7.	25	0.46	24	11.4
8.	12	0.91	13	10.9
9.	12	0.91	13	10.9
10.	12	0.91	15	10.9
11.	12	0.91	17	10.9
12.	16	0.91	18	14.6
13.	16	0.91	18	14.6
14.	16	0.91	18	14.6
		<i>Tartar emetic</i>		
15.	21	0.46	22	9.6
16.	{ 22*	0.46	22	10.0
	{ 2	0.46	1	0.9
17.	{ 8*	0.46	8	3.6
	{ 16	0.46	15	7.4
18.	24	0.46	25	10.9
19.	24	0.46	23	10.9
20.	{ 8*	0.46	8	3.6
	{ 16	0.46	18	7.3
21.	24	0.46	23	10.9
22.	24	0.46	23	10.9
23.	4	0.91	5	3.6
24.	5	0.91	8	4.5
25.	12	0.91	15	10.0

tions were made with particular attention to visual acuity and the presence of nodules presumed to contain adult *Onchocerca*. Skin biopsies were made by the method of Nettel<sup>5</sup> which consists of the microscopic examination of skin snips in saline for the presence of microfilariae. Except in one instance, not less than four such skin samples were examined and the numbers of microfilariae recorded. At the end of the treatment interval and for a period of five to seven months thereafter,

\* We wish to express our thanks to Drs. Bertha Noble and Joseph Spoto and to Mr. Daniel Jobbins who helped in selecting patients and in making the pre- and post-treatment examinations.

skin biopsies were made and visual acuity checked at one- or two-month intervals. Nodules were removed between one and five days after the completion of therapy, a part was dissected for the presence of adult worms, and the remainder was fixed in formalin for histological examination.

Patients were treated with stibophen (Fuadin, Winthrop) and tartar emetic. The first series of 15 patients was treated intravenously with doses of 0.46 mg. of antimony per kilogram of body weight. A single dose was given daily for five days, two doses were given on the sixth day, and no drug was given on the seventh day. A total of 24 or 25 such injections was given. Because no severe reactions occurred with the doubled dose on the sixth day of therapy, a second series of 10 patients was treated with doses of 0.91 mg. of antimony per kilogram of body weight given six days per week for a total dose similar to that of the first series (TABLE 1).

No severe reactions were observed even with the larger doses. There were instances, particularly with tartar emetic, of cough, chest pain, joint pain, antecubital phlebitis, and headache. Two patients had a transitory swelling of the eyelids, and one, of the ears.

Three volunteers were given radioactive antimony.\* However, the quantitative determinations in only one case are pertinent to this paper. This volunteer was given 22 doses of tartar emetic prepared from radioactive antimony.

## RESULTS

After treatment, a total of 33 nodules was removed and examined for the presence of live or dead adult worms. From 9 patients treated with stibophen, 10 nodules contained live adult *Onchocerca*, 4 contained immotile adults, and the examination of one was unsatisfactory. From 11 patients treated with tartar emetic, 8 nodules contained live adult worms, 9 contained immotile adults, and the examination of one was unsatisfactory (TABLE 2).

The visual acuity of 19 patients was checked during the follow-up interval by means of the Snellen chart. Judged by this test, the vision of 12 eyes had improved, 9 had regressed, and in the remainder was unchanged. One patient (No. 2) had had one eye enucleated by Dr. Noble before treatment and could distinguish only lightness and darkness with the other at that time. At the 7-month post-treatment check, this patient could get around without the use of a guide or cane because of improvement in the remaining eye.

TABLE 3 indicates the results of examinations of skin snips for microfilariae made before, during, and after treatment. All were positive before treatment because that was a criterion of selection. Although negative examinations were noted during and after treatment, no trend

\* The antimony was prepared by bombardment in the cyclotron of the Carnegie Institution of Washington, purified, and synthesized into tartar emetic. Quantitative determinations were made as described by Brady *et al.*<sup>6</sup> We wish to express our thanks to Dr. Arthur T. Ness for the chemical preparations.

TABLE 2

THE RESULTS OF GROSS EXAMINATION FOR MOTILITY OF ADULT *Onchocerca* ONE TO FIVE DAYS AFTER TREATMENT (THE MINUS SIGN INDICATES IMMOTILITY)

Patient No.	Nodules		
	1	2	3
<i>Stibophen</i>			
1.	+	+	
2.	+	—	
3.	—		
4.	+	+	+
7.	?		
8.	+		
9.	—	—	
12.	+		
13.	+	+	
<i>Tartar emetic</i>			
15.	+	—	
16.	+	—	
17.	+		
18.	—	—	
19.	+	+	
20.	—		
21.	?		
22.	+	—	—
23.	+		
24.	—		
25.	+	—	

could be discerned, the number negative at 6 and 7 months post-treatment checks being similar to the number negative at the mid-treatment check.

In the case of patient No. 16 who was given radioactive tartar emetic, two nodules were extirpated 28 days after the beginning of therapy and 6 days after the last injection of radioactive material. In addition, a wedge of apparently normal subcutaneous tissue with a small amount of skin was removed. Adult *Onchocerca* were dissected out of one nodule. These samples were dried and examined for their content of antimony. The following results were obtained:

Adult filariae: 12.0 micrograms of antimony per gram of dry weight.

Nodule containing filariae: 4.4 micrograms of antimony per gram of dry weight.

Subcutaneous tissue: 0.8 micrograms of Sb per gram of dry weight.

To determine whether or not treatment with antimony had had any effect on the adult *Onchocerca*, nodules containing the worms were excised, fixed in 10 per cent formalin, and after paraffin embedding, sectioning, and azure cosinate staining, were examined microscopically for evidence of degenerative changes. Twenty-two nodules from 16 treated patients were studied. Nodules were removed from 1 to 5 days following completion of treatment. In order to have a basis for determining the significance of any degenerative changes found, a series of 19 "control" nodules was obtained from untreated patients and similarly

TABLE 3  
EXAMINATIONS OF SKIN BIOPSIES FOR MICROFILARIAE;—DENOTES NO  
MICROFILARIAE FOUND IN AT LEAST 4 SKIN BIOPSIES

Patient No.	Biopsy							
	Pre-treatment	Mid-treatment	Post-treatment					
			1 mo.	3 mos.	4 mos.	5 mos.	6 mos.	7 mos.
<i>Stibophen</i>								
1.	+	+	+	+	+	+		+
2.	+	+	+	+	+	+		+
3.	+	+	+	+	—			+
4.	+	—	+	+	+	+		+
5.	+	+	+	+	+	+		+
6.	+	+						
7.	+	+	+	+	—	+		+
8.	+						+	
9.	+	+			—		—	
10.	+	+			+		+	
11.	+	+						
12.	+	+			—		+	
13.	+				—		+	
14.	+	+			—		+	
<i>Tartar emetic</i>								
15.	+	—	—	+	+	—		—
16.	+	+	+	+	+	+		+
17.	+	+	+		+	+		—
18.	+	+	+	+	+	+		+
19.	+	—	+	—	—	+		—
20.	+	+	+	+	+	+		+
21.	+	—		+				
22.	+	+	+	+	+			
23.	+				+		+	
24.	+				+		+	
25.	+				+		—	

examined. A previously reported study of *Dirofilaria immitis*<sup>1</sup> served to direct particular attention to the genital tract of the female *Onchocerca*.

In view of the fact that both the control and treated series are small and since the results of the microscopic examination do not permit a definite conclusion as to the effect of treatment on the adult worms, details of the histopathologic findings in each case will not be given. Only general statements and comparison of the findings in the two groups will be made.

Degenerative changes involving the contents of the uteri of the adult worms with or without marked suppression of microfilaria production were seen in both the treated and untreated series. The changes observed were qualitatively similar. For purposes of comparison, the female worms of each nodule were classified into one of three groups, as follows:

*Group 1.* The worms placed in this category showed developmental forms in the proper uterine level and morphologically differentiated micro-

filariae in the anterior portion of the uteri. Some worms showed slight variation from normal in the tinctorial quality of the cytoplasm of the cells of the developing and differentiated microfilariae. Since the significance of this minor change was not clear, these worms were considered essentially normal and placed in this group.

**Group 2.** The majority of the worms in this group showed both developmental stages and differentiated microfilariae. However, in all of them, there was karyorrhectic or frank necrosis involving some of the developmental stages in various uterine levels. Rarely, the entire contents of the uterus of one level were necrotic. In a very few worms placed in this group, there were no microfilariae.

**Group 3.** In this group were placed those worms which showed complete suppression of microfilarial production and absence of developing stages. The uteri of the worms of this group, at most, contained a few undeveloped and often degenerating ova. One worm was dead and partly calcified and two others showed completely empty uteri.

The ovaries were seen in only a few worms. No clear-cut changes were evident.

Using the criteria outlined above, for separating the nodules into three groups, a comparison of the findings in the treated and untreated patients is made in TABLE 4. It is evident, as mentioned earlier, that *Onchocerca*

TABLE 4  
COMPARISON OF HISTOPATHOLOGIC FINDINGS IN FEMALE *Onchocerca* IN  
UNTREATED PATIENTS AND PATIENTS TREATED WITH ANTIMONY

Group* (See text for details)	Number of nodules from untreated patients	Number of nodules from treated patients
1	13	8
2	2	7
3	4	7

\* Group 1—Essentially normal worms;

Group 2—Reduced reproductive function with definite degenerative changes;

Group 3—Absence of reproduction.

from untreated patients may show definite, sometimes marked, degenerative changes and suppression of microfilaria production. This makes interpretation of the results of the study most difficult and necessitates the comparison only on an incidence basis. This comparison indicates that treatment in some cases had a deleterious effect on the reproductive function of the adult filariae. This statement is based on the fact that, when compared to the "controls," the number of normal worms (group 1) in the treated patients, was reduced and that in groups 2 and 3 was increased. The cautious wording of the results shown is dictated by the small series of cases and the fact that degenerative changes or suppression of microfilaria production occurred in about 1/3 of the worms from untreated patients.

## DISCUSSION

The treatment of onchocerciasis with trivalent antimonials has been reported by several investigators,<sup>7-9</sup> but no evidence of benefit was found.

In an unpublished study, two of the present authors (F. C. Bartter and F. J. Brady), with Dr. Luis Figueroa Ortiz, treated in 1944 a total of 16 cases with three trivalent antimony compounds: Stibophen,\* *p*-phenetidine antimonyl tartrate, and sodium antimonyl xylitol. These patients each received a total of 3.5 mg. of antimony per kilogram of body weight over an interval of 15 days. Objectively, there was no improvement after treatment because skin and nodule biopsies showed motile microfilariae and adults, respectively, after treatment, and there was no significant change in complement fixation titers when performed by the method recorded by Bozicevich *et al.*<sup>10</sup> Subjectively, 6 patients claimed that their *Onchocerca* nodules had diminished in size with treatment and one patient who was able only to distinguish lightness from darkness before treatment could count fingers after treatment.

The evidence presented in the study reported here does not indicate any effect of stibophen or tartar emetic on the microfilariae. It was felt that the six to seven months' observation period should be adequate to detect any lethal effect on the microfilariae, particularly in view of the rapid disappearance of microfilariae of other species of filarids in the dog and man following therapy with trivalent compounds.

The results of studies of the adult parasites after treatment are encouraging. There are available data which permit a comparison of the antimony content of the worms dissected from a nodule of patient No. 16 and those of a dog successfully treated for *Dirofilaria immitis*.<sup>11</sup> This dog was given a total of 9.6 mg. of antimony per kilogram over an interval of 14 days. The live parasites had an antimony content of 11.0 micrograms and the subcutaneous tissue contained 0.4 micrograms per gram of dry weight 36 hours after the last injection. This dog was free of circulating microfilariae after the ninth injection. In the volunteer with onchocerciasis, a total of 10.0 mg. of radioactive antimony was given per kilogram and the amount in the adult worms and subcutaneous tissues was very similar to that in the case of the dog. Not only is a specific uptake of antimony by adult *Onchocerca* indicated, but the antimony must reach the adult worms within the connective tissue in quantities that are within the effective range. There is, however, no indication of the chemical state of the antimony.

The adult parasites may be morphologically altered by the treatment, though the present work does not unequivocally indicate such changes. The period following treatment until extirpation of the nodules, 1 to 5 days, was too short for a suitable evaluation of such changes. Degenerative changes sometimes are present in female *Onchocerca* from untreated individuals and a series larger than ours would be needed to show significant differences.

\* Provided through the courtesy of Winthrop Chemical Co., Inc., New York, N. Y.



## SUMMARY

Twenty-five patients with onchocerciasis were treated with stibophen and tartar emetic in doses of 0.46 and 0.91 mg. of antimony per kilogram of body weight, the majority receiving a total of 11 mg. per kilogram. Examination of post-treatment skin and nodule biopsies did not reveal any significant decrease in the numbers of microfilariae or in the motility of adult parasites.

The use of tartar emetic prepared from radioactive antimony in one case permitted a measurement of the antimony uptake by the adult parasites present in one nodule. The uptake was similar to that of adult *Dirofilaria immitis* recovered from a dog successfully treated for filariasis.

There was some evidence that adult *Onchocerca* females were injured by the therapy, but the data do not permit definite conclusions.

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## TOLERANCE OF ANTIMONY AND ARSENIC BY INTENSIVELY TREATED PATIENTS\*

By HARRY M. ROSE †

*Departments of Medicine and Bacteriology, College of Physicians and Surgeons,  
Columbia University, New York, N. Y.*

Among the most important properties of antimony are the toxic reactions it produces. The deleterious effects of this element have been well recognized since the ancients employed it both as a medicine and as a cosmetic, and today our attention continues to be drawn to the toxic reactions of greater or less severity induced by the parenteral administration of organic compounds of antimony which are widely employed for the treatment of human infections caused by protozoan and helminthic parasites. The untoward symptoms which are produced during the therapeutic use of antimony compounds are well known. In the main, they consist of nausea, vomiting and, occasionally, diarrhea, all of which commonly appear shortly after injection, particularly if the drugs are given by the intravenous route. Malaise, headache, anorexia, and fever are not unusual. Severe bouts of coughing may be seen after intravenous administration. Pains in the joints and muscles are common complications, and joint involvement simulating acute arthritis appears from time to time. Salivation is not infrequently seen. In some cases, following prolonged administration, skin eruptions accompanied by desquamation and a good deal of itching occur. The effect of antimony on the cardiovascular system may result in marked slowing of the pulse, a fall in blood pressure, and in some cases may produce an acute shock-like syndrome necessitating immediate supportive therapy. Damage to the liver and especially to the kidneys with resulting hepatic and renal insufficiency have been observed. Toxic suppression of the activity of bone marrow occurs very rarely.

Because of their side effects which may be severe and even fatal, the administration of organic compounds of antimony is ordinarily conducted with due caution and respect. In spite of the inherent dangers attending their use, a great mass of evidence now attests their value in the therapeutics of a number of parasitic diseases.<sup>1</sup> It is of interest to us, however, and germane to the present discussion that in most of the protozoan and helminthic diseases in which antimony compounds are employed for treatment, the drugs are usually administered in relatively moderate dosage, chiefly because of their well-known tendency to excite side reactions which are either troublesome or sufficiently severe as to necessitate their discontinuance.

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† In collaboration with James T. Culbertson, University of Arkansas, School of Medicine, Little Rock, Arkansas, and José Oliver-Gonzalez, Federico Hernandez Morales, and Caroline Kreiss Pratt, School of Tropical Medicine, San Juan, Puerto Rico.

Certain antimony compounds are effective chemotherapeutic agents in the treatment of filarial infections in both animals<sup>2</sup> and humans.<sup>3-7</sup> However, it is evident that, in order to obtain satisfactory results, doses rather larger than those ordinarily used are necessary. In the course of a study on the chemotherapy of filariasis, patients were treated with a number of different antimonial drugs.<sup>8</sup> The drugs employed were neostibosan, neostam, fuadin, anthiomaline (Specia), anthiomaline (Merck), urea stibamine, tartar emetic, and stibanose. Seventy-five patients were intensively treated with these compounds and the plan of therapy in each group of patients was to give a single course of the drug, usually over a period of two weeks, administering each drug to the limits of tolerance. Toxic reactions of varying character and severity were noted to accompany the administration of each compound. The reactions to some of the drugs were relatively inconsequential, in spite of the continued administration of large daily doses, whereas with other drugs the reactions were frequent and severe and in many cases made it necessary either to modify the dosage schedule or entirely to discontinue treatment. The most striking phenomenon in this series of intensively treated patients was the development of tolerance to certain compounds, notably neostibosan, and the progressive appearance of intolerance to others of the drugs that were

		REACTIONS TO NEOSTIBOSAN													
Patient	Total Drug gms.														
1	7.9														A-ABDOMINAL PAIN
2	12.5				NV										C-CONJUNCTIVITIS
3	5.7		NV		NV										F-FEVER
4	11.5														H-HEADACHE
5	9.0	AH													N-NAUSEA
6	12.0														V-VOMITING
7	11.1														
8	12.0														
9	10.0														
10	12.5				NV		NV								
11	11.0		NV												
12	9.5	NV													
13	15.5					F	F	F	F						
14	11.0	V								C	C	C	C	C	C
15	12.3		NV	H	C	C	C	C	NV						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Days													

FIGURE 1.

employed. It is the purpose of this discussion to describe the nature and frequency of toxic reactions to the eight antimonial compounds already referred to, and especially to note the chronological sequence of these reactions. In addition, we wish to note the level of tolerance to a certain trivalent arsenic compound, melarsen oxide, and to describe an experimental study of the tolerance of pentavalent antimony by patients who had previously received trivalent antimony, as well as the development of tolerance of patients who had received a course of treatment with one pentavalent compound to another pentavalent compound.

FIGURE 1 illustrates the reactions that were observed in a group of fifteen patients intensively treated with neostibosan intravenously. The patients received total doses of drug ranging from 5.9 grams to 15.5 grams over a period of two weeks. Six of these individuals experienced no type of reaction whatever. In the others, nausea and vomiting were the chief and usually the only symptoms, and these ordinarily appeared within 10 to 20 minutes after the drug had been injected. It is important to note that reactions, when they occurred, were seen early in the course of therapy, but, as relatively large doses of the compound continued to be administered each day, in nearly all cases the signs of toxicity tended to disappear, so that the latter period of therapy usually was accomplished

		REACTIONS TO NEOSTAM													
Patient	Total Drug gms.														
1	10.6	NV	N	ANV	N	N	HN	AN	FR	FR	FR	FR	FR	FR	FR
2	11.4	FH	F	F	F	F	HFR	FH NR	FRV	FAR	FR	FR	FR	FR	FR
3	10.6	NV	NV		NV			FR	FAR	FR	FR	CFR	CFR	FR	FR
4	7.7		HNV	HNV											
5	9.85	NV	NV	ANV	NV	ANV	NV	FN VR	FN VR	FR	FR	FR	FR	FAR	FR
6	8.5		NV	NV											
7	10.85	NV		ANV		H	ANV	FR	FR	FR	FR	FR	CFR	ACF	CF
8	11.0	NV	A	NV		H		FR	FR	FR	FR	FR	FR	F	FNV
9	10.1	ANV	NV	NV	F	F	F	FR	FR	FR	FR	FAR	F	F	NV
10	8.7		NV				A								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Days													
		A - ABDOMINAL PAIN							H - HEADACHE						
		C - CONJUNCTIVITIS							N - NAUSEA						
		F - FEVER							R - RASH						
		V - VOMITING													

FIGURE 2.

without incident. The pattern suggests definitely that, as treatment with ncostibosan is continued, a very considerable degree of tolerance to this compound is developed. On this account, although in some of these patients really formidable amounts of the drug were administered, in no case was it ever necessary to discontinue treatment because of toxic reactions.

Ten patients were treated with ncostam (FIGURE 2), receiving three injections of the drug daily by the intravenous route. The total amounts of drug administered over a three-week period of treatment ranged from 7.7 grams to 11.4 grams. Severe toxic reactions were seen in nearly every patient throughout the period of treatment. During the first week of therapy, the chief reactions observed were nausea and vomiting immedi-

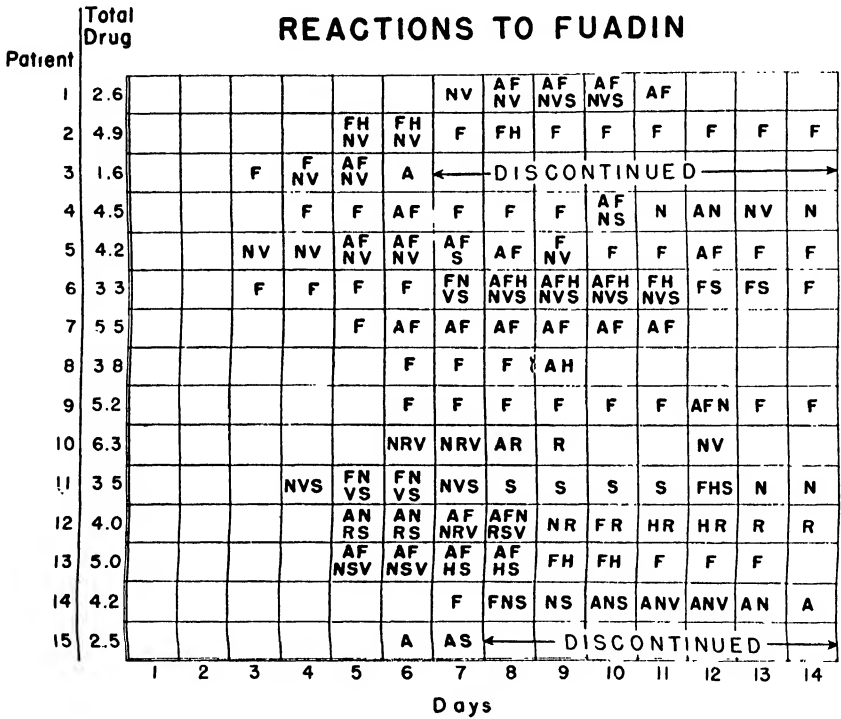


FIGURE 3.

ately following injection of the drug. As administration was continued and the doses of the drug were increased, nausea and vomiting tended to disappear, illustrating the acquisition of a certain amount of tolerance. However, during the second week of therapy, although nausea and vomiting were observed in only one or two instances, nearly all of the patients developed some fever together with skin eruptions characterized by erythema, vesiculation, desquamation, and more or less marked itching. In spite of these manifestations, drug therapy was continued in all patients

and the courses of treatment were successfully completed, although in some cases the total amounts of drug administered were less than had originally been planned. While signs of damage to the liver, the kidneys, and the bone marrow did not appear, it was difficult to keep patients under therapy with neostam because of the other forms of toxicity, and we consider that the administration of large amounts of this compound in a program of intensive therapy is potentially hazardous and not to be recommended.

As shown in FIGURE 3, fifteen patients were treated with fuadin intramuscularly for fourteen days, receiving total doses ranging from 1.6 to 6.3 grams. No reactions were seen in any patient until the third day, after which, however, they were seen in every case. The reactions were of such

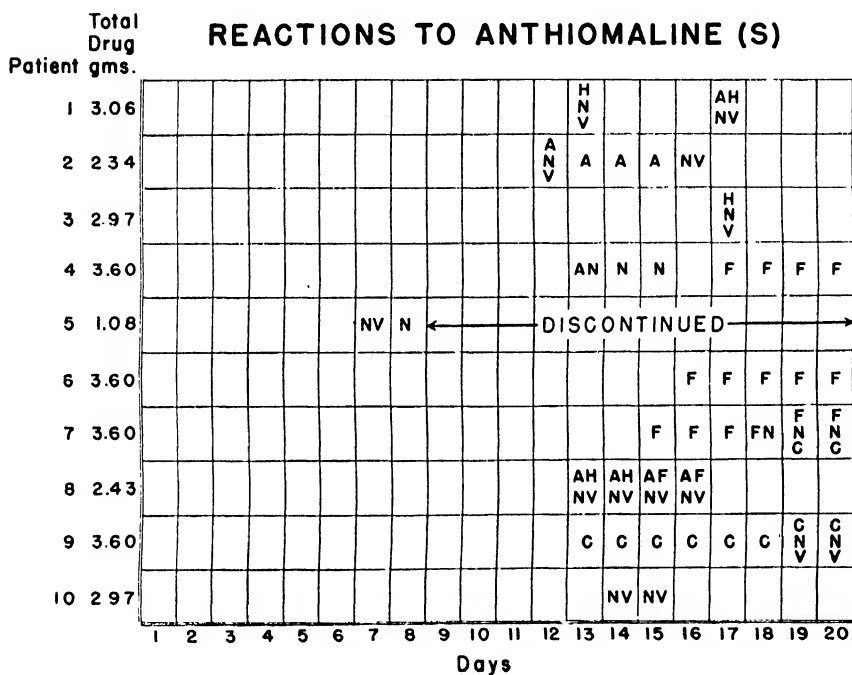


FIGURE 4.

severity that in no case could the planned program of therapy be carried out, and in two patients the drug had to be discontinued permanently after seven and eight days, respectively. The symptoms of toxicity that were observed consisted primarily of anorexia, fever, headache, nausea, vomiting, and salivation. In one patient a skin eruption appeared. It is of considerable interest that the signs of toxicity were not observed immediately following the initial administration of this drug, but tended to develop slowly and, in some individuals, were not observed until after the first week of therapy. In all patients, however, once toxic signs or

symptoms made their appearance they continued to become progressively severe when an attempt was made to maintain the drug in full dosage, and the only way treatment could be continued for a total of two weeks, in nearly all instances, was by progressively decreasing the daily dose. It is apparent that, in dealing with fuadin, we were confronted with the progressive development of an intolerance to the drug. This is quite the reverse of what we encountered in the patients treated with neostibosan.

FIGURE 4 shows the results in ten patients treated with anthiomaline (Specia) intramuscularly in total dosage from 1.08 to 3.60 grams. The plan of treatment in this group called for a daily single injection of 0.18 grams in a total volume of 3 ml. over a period of 20 days. As with fuadin, the initial days of the therapeutic program were completed without incident and, in nine of the ten patients, no reactions whatever were seen until the 12th day. Thereafter, however, reactions occurred and

		REACTIONS TO ANTHIOMALINE (M)													
Patient	Total Drug gms.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	3.36					N	NS	NS	ANS	A	A				
2	4.20						S	RS	RS	RS	RS	RS	S	S	
3	2.94				R	R	FRS	FRS	ANV FRS	ANV FRS	ANV FRS	ANV FRS	ANV FRS	ANV FRS	ANV FRS
4	2.04				F	F	FR	FR	FR	R	R				
5	2.76				AR	AR	AR	ANV FRS	ANV FRS	ANV FRS	← DISCONTINUED →				
6	2.28				F	F	F	FR	FR	FRS	HRS	HRS	HRS	HRS	HRS
7	1.68						NVR	NVR	NVR	← DISCONTINUED →					
8	2.70						F	F	F	F	F				
9	1.92						NS	NS	NS	NS					
10	2.34							ANS	ANV S	ANS	ANV S	ANS	ANS	ANS	ANS

FIGURE 5.

became progressively severe. In one patient, whose first toxic reaction occurred on the seventh day, treatment with the drug had to be discontinued permanently. It is obvious that with anthiomaline, as with fuadin, the patients displayed a progressive intolerance to the drug.

FIGURE 5 illustrates the reactions in ten patients treated with anthiomaline (Merck) intramuscularly over a period of fourteen days, receiving total doses from 1.68 to 3.36 grams. This drug was administered in as large a dosage as was possible. Again, as with anthiomaline (Specia), the toxic reactions were delayed in their appearance and none of the patients experienced any ill effects until the 4th day. Thereafter, however, the

reactions became progressively severe and in two patients the drug had to be discontinued on the 9th and 10th days, respectively. Due to the fact that the patients developed, in most cases, such a marked intolerance to the drug as treatment was continued, it was impossible to continue the daily administration of large doses and the two-week therapeutic program could only be finished by decreasing the daily doses of the compound and sometimes omitting them entirely. Consequently, the total amounts of drug received were relatively small in eight of the ten cases.

Patient	Total Drug gms.	REACTIONS TO UREA STIBAMINE													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
B	1 4.2				AH NV	AC HN	AC HN	AC HN	AC HN	AC HN	ACH NVS	HNS	HNS	AH NS	HNS
	2 3.3			AH NV	A	AH	A	A	A	AH NV	AHN	AHN	AH NS	AH NS	AH NS
	3 4.95			AH NV		AH			H	HS					
S	4 6.8					H	H	H							
	5 7.1														
	6 7.1										H				

		REACTIONS TO TARTAR EMETIC													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.77			A	A						AR	R	R	R	AR
2	0.73								R	R	HR	HR	HR	HR	HR
3	0.79								R	R	R	HR	R	R	R
4	0.88								R	R	AR	R	HR	HR	R

FIGURE 6.

Six patients were treated with urea stibamine over a period of two weeks, as shown in FIGURE 6, receiving total doses ranging from 3.3 grams to 7.1 grams. The six patients were divided into two groups of three each, one of which received the Brahmachari preparation of the drug, and the other group the compound as prepared by E. R. Squibb & Sons. Reactions to the Indian preparation were quite severe in every instance, necessitating the administration of small individual doses, and it appeared that more intensive therapy with this preparation was distinctly dangerous. On the other hand, the Squibb preparation of urea stibamine was very well tolerated. Two of the patients experienced a little headache, but otherwise no reactions of any consequence were noted.

Four patients were treated with tartar emetic intravenously over a period of two weeks in total dosage ranging from .73 to .88 grams (FIGURE 6).



Relatively small doses of this compound were administered because of its known toxicity, but in spite of this precaution all of the patients experienced reactions late in the course of therapy. It should be noted once more that with tartar emetic, as with fuadin and anthiomaline, the administration of trivalent antimony did not result in early symptoms or signs of toxicity, but these developed toward the end of the first week and became progressively severe.

Five patients received stibanose intramuscularly three times daily for two weeks in a total dose ranging from 13.87 to 15.21 grams. No reactions of any sort were encountered in this group.

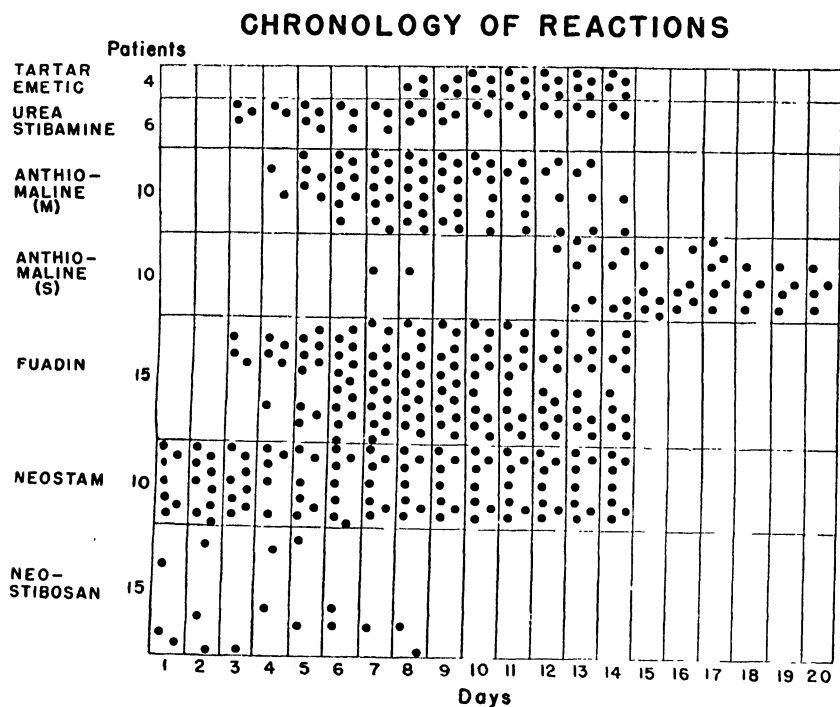


FIGURE 7.

In addition to the seventy-five patients that were treated with the antimonial compounds, eighteen patients were also given a course of therapy with a trivalent arsenical preparation, mclarsen oxide. This drug is available in capsules for administration by mouth and can also be given intravenously dissolved in propylene glycol. Fifteen of the patients received the drug by the intravenous route. Seven were given daily doses of 7.5 milligrams over a period of one week. No toxic reactions of any kind were observed during the period of therapy, but one patient on the day following the completion of treatment developed mild headache and a low-grade fever which persisted for 48 hours. Eight patients

were then given slightly larger doses of the drug, receiving 10 milligrams daily by the intravenous route over a planned period of ten days. However, on the eighth and ninth days, respectively, two of the patients suddenly developed a severe form of toxic encephalitis. For several days both were critically ill, but fortunately they recovered eventually without permanent residual effects. Two other patients experienced malaise and moderately severe headache without, however, any encephalitic manifestations. The remaining four patients of this group had no toxic reactions whatever. Three patients were given mclarsen oxide by mouth in doses of 50 milligrams three times daily for periods of 8 to 14 days. Two of these individuals showed signs of toxicity as evidenced by malaise and fever, and in addition one of them developed a toxic erythema. As a result of these experiences, it was concluded that mclarsen oxide is potentially too dangerous for use even in the rather moderate doses that were administered.

A noteworthy observation on the response of patients to antimony compounds administered intensively by parenteral routes, was the chronological sequence of the reactions and the remarkable development of tolerance to neostibosan (FIGURE 7). Reactions with the pentavalent preparations, neostibosan and neostam, appeared early in the course of treatment. With neostibosan, these reactions were mild and tended to disappear, or disappeared completely, in spite of the continued administration of large doses of the drug. With the trivalent antimony preparations, on the other hand, reactions were never seen before the third day of treatment, but thereafter they regularly became increasingly severe and necessitated the cessation of therapy in a number of patients.

TABLE 1

REACTIONS TO NEOSTAM FOLLOWING THERAPY WITH TARTAR EMETIC  
AND ANTHIOMALINE

Case	Previous therapy		Provocative test	
	Drug	Amount	Neostam 50 mg.	Neostam 150 mg.
1	Tartar emetic	0.88 gm.	No reaction	Severe abdominal pain
2	Tartar emetic	0.79 gm.	No reaction	Severe abdominal pain
3	Tartar emetic	0.85 gm.	Abdominal pain Diarrhea	Not given
4	Tartar emetic	0.73 gm.	No reaction	Severe abdominal pain Nausea and vomiting Shock requiring treatment
5	Anthiomaline	2.94 gm.	Diarrhea	Severe abdominal pain Nausea and vomiting
6	Anthiomaline	4.20 gm.	No reaction	Nausea and vomiting
7	Anthiomaline	2.76 gm.	No reaction	Nausea and vomiting

A study was made to determine whether the administration of trivalent antimony would induce tolerance to pentavalent antimony. One group of three patients at the end of their course of therapy with anthiomaline, and another group of four patients treated with tartar emetic, both trivalent preparations, were given small doses of neostam, a pentavalent compound. The results are shown in TABLE 1. A small provocative dose of 0.05 gm. of neostam intravenously caused diarrhea and abdominal pain in two of the seven patients within half an hour after injection. The five patients who showed no effects from the first dose of neostam were then given a second dose of 0.15 gm. three hours later. Reactions characterized by severe abdominal pain, nausea, and vomiting were seen in every instance within twenty minutes, and one patient exhibited a shock-like syndrome requiring immediate supportive therapy. Doses of neostam below 0.2 gm. invariably produced no reactions in patients who were completing their course of intensive treatment with this drug. It thus became apparent that the previous administration of the trivalent antimony compounds did not raise the level of the patients' tolerance to small doses of the pentavalent compound.

Another group of five patients, at the end of their course of treatment with neostam, were given single injections of 0.3 or 0.5 gm. of neostibosan, which also contains pentavalent antimony. As shown in TABLE 2, no

TABLE 2  
REACTIONS TO NEOSTIBOSAN FOLLOWING THERAPY WITH NEOSTAM

Case	Previous therapy		Provocative test		
	Drug	Amount	Drug	Amount	Reaction
1	Neostam	9.85 gm.	Neostibosan	0.3 gm.	None
2	Neostam	10.85 gm.	Neostibosan	0.3 gm.	None
3	Neostam	11.40 gm.	Neostibosan	0.3 gm.	None
4	Neostam	10.60 gm.	Neostibosan	0.3 gm.	None
5	Neostam	10.60 gm.	Neostibosan	0.5 gm.	None

reactions occurred in any of these patients, although, in our experience, neostibosan given intravenously in doses exceeding 0.2 gm. always caused nausea and vomiting in patients who received it for the first time. The failure of the neostam-treated patients to react to the large doses of neostibosan must indicate, therefore, that the administration of the one pentavalent drug induced tolerance of the other.

### SUMMARY

The intensive administration of organic antimony compounds by the intravenous or intramuscular route is accompanied by toxic side reactions of varying character and severity. With some preparations, notably neo-

stibosan, the continued administration results in the development of considerable tolerance, with progressive diminution in severity or even complete disappearance of toxic side effects. With other compounds, however, notably those containing trivalent antimony, continued administration results in progressively greater intolerance, with increasingly severe side reactions, so that dosage schedules have to be modified or the drugs discontinued entirely.

There is evidence that the administration of trivalent antimony will not result in any increase in tolerance of pentavalent antimony. However, the administration of one pentavalent preparation appears to produce an increase in tolerance to another pentavalent drug.

The trivalent arsenical compound, melarsen oxide, may produce dangerous toxic reactions when given in moderate dosage.

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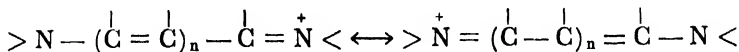
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# CHEMISTRY OF THE CYANINE DYES\*

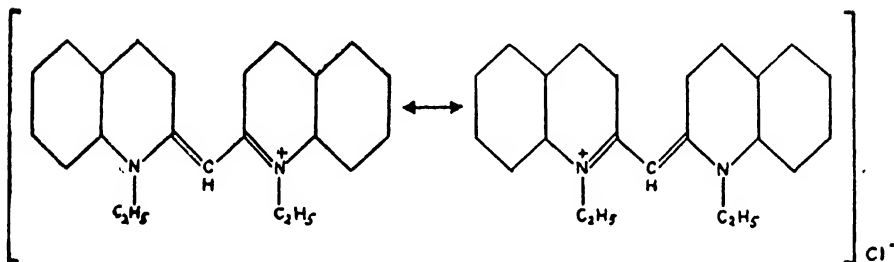
By L. G. S. BROOKER

Eastman Kodak Research Laboratories, Rochester, N. Y.

Several families of basic dyes are characterized by the possession of the amidinium system, the essential grouping of which is:



When the two nitrogen atoms are members of heterocyclic rings and the unsaturated chain between them also passes through these rings, such dyes are known as *Cyanines*. These dyes, in general, are not fast to light and their chief interest has been in photography, where for a good many years they have been of outstanding value as color sensitizers. Methods of synthesis have been elaborated that have provided cyanines differing widely in structure, absorption, and solubility. Where the two nuclei comprising the cyanine are identical, the dye is said to be *symmetrical*, and an example is provided by the dye following, the nuclei of which are derived from quinoline:



The  $=\text{CH}-$  group between the quinoline rings in this dye may be increased in length to  $=\text{CH}-\text{CH}=\text{CH}-$ ,  $=\text{CH}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$ ,  $=\text{CH}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-$  or even to longer lengths and these chains may furthermore be substituted at certain positions. The ethyl groups shown on the nitrogen atoms may be replaced by other alkyl or aralkyl groups and the quinoline rings may themselves be substituted, or replaced by a wide variety of other heterocyclic rings. Still other cyanines may be obtained by combining rings of two different kinds to give *unsymmetrical* cyanines. It is clear that the number of possible cyanines is, thus, very great indeed. Theoretical considerations lead to the conclusion that, wherever possible, the cyanine molecules will have a planar configuration but in certain cases planarity must be impossible.

\* For further details the reader is referred to such review articles as Chapter XXIV in Mees's *Theory of the Photographic Process* (Macmillan, New York, 1942); and *Steric Hindrance to Planarity in Dye Molecules*, Chem. Rev., October, 1947.

# CHEMOTHERAPEUTIC ACTIVITY OF CYANINES AND RELATED COMPOUNDS IN FILARIASIS IN THE COTTON RAT\*

By HAROLD N. WRIGHT, JOHN T. LITCHFIELD, JR., THERESA BREY,  
ELIZABETH M. CRANSTON, ASHTON C. CUCKLER, AND  
RAYMOND N. BIETER

*Department of Pharmacology, University of Minnesota Medical School,  
Minneapolis, Minnesota*

A group of more than sixty cyanine dyes and related compounds has been studied<sup>1-3</sup> for chemotherapeutic activity against the naturally acquired *Litomosoides carinii* infestation in the wild cotton rat as part of a coordinated research program on filariasis under the auspices of the Office of Scientific Research and Development. A similar study with a group of pyrrolyl, pyrimidyl and related cyanines was carried out simultaneously by Dr. A. D. Welch and his associates<sup>4-9</sup> at Western Reserve University.

Care of the animals and administration of drugs was greatly facilitated by the use of special cages, holders, and feed cups designed by one of the authors (J. T. Litchfield, Jr.). The cages were made of a heavy wire mesh, were oblong in shape and of a size suitable for housing the rats individually. One end of the cage was designed as consisting of flanged sheet metal into which fitted a sliding door and to which either the holder or feed cup could be attached (FIGURE 1). The holders were equipped with sliding doors at both ends and had a curved piece of metal extending most of the length inside the top, which was attached by springs to a metal bar outside the holder. With the holder in hand, slight pressure from the palm could be exerted on the bar, thus pressing the abdomen of the rat gently against the wire mesh bottom of the holder, through which intraperitoneal injections were then made. For drug diet administration, feed cups were attached to the metal flanges on the outside of the cages, thus allowing easy removal for daily filling and weighing.

Tests for chemotherapeutic activity were made *in vivo* against the adult filaria. Administration was usually by intraperitoneal injection every eight hours for eighteen doses with autopsy on the eighth day. The filaria were removed at autopsy, placed in a modified Sim's solution and examined for motility for 24 hours. The minimum curative dose was taken to be that dose of drug which killed 50 per cent or more of the adult filaria. Therapeutic indices were determined as the ratio of the maximum tolerated dose (M.T.D.) to the minimum curative dose (M.C.D.) based

\* This work was done under contract with the Office of Scientific Research and Development, the Office of the Surgeon General of the R. S. Army, the United States Public Health Service, and the University of Minnesota. Grateful appreciation is made to the Eastman Kodak Company and Parke, Davis and Company for supplying these compounds through the Chemotherapy Center.

on the graded dosages used which usually were separated from one another by a factor of three. For this reason, the therapeutic indices are given as a range of activity, the lower figure representing the established minimum, the higher the possible maximum therapeutic index.

The majority of compounds tested were styryl quinolines or diquinolines. In the group of *p*-amino styryl quinolines (FIGURE 2), 2-*p*-diethylamino styryl-6 methyl quinoline, containing two trivalent nitrogens, showed only questionable activity, whereas the ethyl chloride homologue, 2-*p*-diethyla-

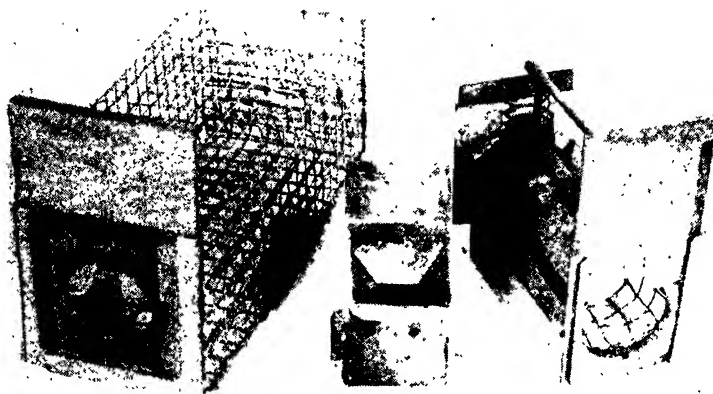


FIGURE 1.

mino styryl-6-methyl quinoline etho-chloride, gave a therapeutic index of 6-10, thus demonstrating a marked increase of activity when one pentavalent and one trivalent nitrogen were present. All *p*-amino styryl quinolines with one pentavalent and one trivalent nitrogen tested (twenty in number) showed activity with therapeutic indices ranging from 1-3 to 9-27. Activity was entirely lost if the amino group on the styryl ring was removed. Increasing the carbon chain on the quinoline nitrogen did not seem to alter activity. Substitutions in position six of the quinoline ring were not necessary, but some evidence was obtained to indicate that increasing the carbon chain of alkyl groups in this position enhanced activity. The irritant properties of the compounds, however, increased faster than the therapeutic activity. All the compounds tested in this series had alkyl substitutions on the *p*-amino nitrogen of the styryl ring.

Propyl groupings here have greater activity than methyl, ethyl, butyl or amyl groups, the diisopropyl being better than the di-*n*-propyl. The most active compound in the series of styryl quinolines tested was 2-*p*-diisopropyl amino styryl-6-methyl quinoline methochloride, which was effective in a dose of 0.1 mg. per kg. for eighteen doses and gave a therapeutic index of 27.

Three vinyl pyridine compounds were tested, one of which showed slight activity, the other two giving entirely negative results. Six quinoline derivatives with alkyl, alkoxy, mercapto or amino substitutions gave little or no activity. Some of these compounds contained a trivalent nitrogen

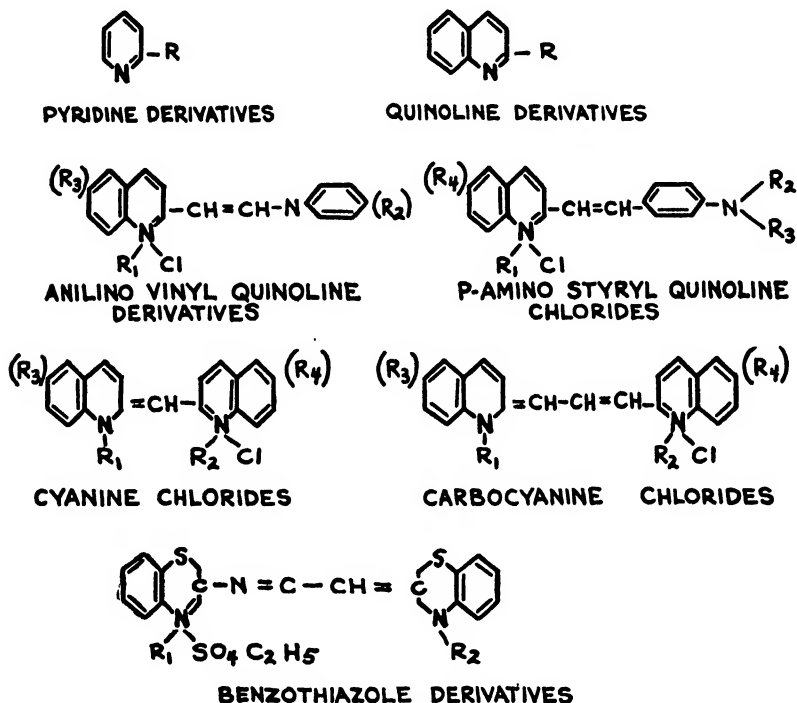


FIGURE 2.

and some a pentavalent nitrogen in the pyridine or quinoline ring. Two derivatives of anilino vinyl quinoline and one anilino methylene quinoline were negative.

The group of diquinoline compounds tested included five cyanine chlorides, eleven carbocyanine chlorides, and a number of compounds having a related structure such as the benzothiazoles (FIGURE 2). All of the compounds tested in the cyanine and carbocyanine series possessed some degree of therapeutic activity.

The parent substance of the series 1,1'-dimethyl 2,2'-cyanine chloride possessed only minimal activity. Substitution of methoxy groups in the



6,6' positions slightly enhanced the activity. The *mono*-benzo derivative, 1,1'-dimethyl 3,4-benzo 2,2'-cyanine chloride, however, possessed considerable activity, having a therapeutic index of 9-27. An equal degree of activity was encountered when the two quinoline molecules were linked together through the 4 position as in 1,1'-dimethyl 4,4'-cyanine chloride (therapeutic index 9-27).

The carbocyanines, in general, were more potent than the corresponding cyanines. The carbocyanine analogue, 1,1'-dimethyl 2,2'-carbocyanine chloride (therapeutic index 3-9), was approximately nine times as potent as the parent cyanine, 1,1'-dimethyl 2,2'-cyanine chloride. The same degree of therapeutic activity was possessed by the corresponding 4,4'-carbocyanine, 1,1'-dimethyl 4,4'-carbocyanine chloride. Therapeutic activity was depressed by a 2,4'-carbocyanine linkage, by substitution of methoxy groups in the 6,6' positions, and by a 3,4,3',4' di-benzo substitution.

On the other hand, the therapeutic activity was enhanced by replacing the methyl groups on the nitrogen atoms with ethyl groups, as in 1,1'-diethyl 2,2'-carbocyanine chloride (therapeutic index 9-27), and was tremendously enhanced by substituting ethoxy groups in these ethyl groups to produce the compound 1,1'-di-B-ethoxyethyl 2,2'-carbocyanine chloride and its *p*-toluene sulfonate salt (therapeutic indices 80-160).

Attempts to modify this structure further gave only compounds of less activity, as for example the 6,6'-dimethoxy derivative and the 1,1'-di-B-phenoxyethyl derivative, both of which, while active, had therapeutic indices of only 9-27.

The cyanine compound 1,1'-di-B-ethoxyethyl 2,2'-carbocyanine chloride and its *p*-toluene sulfonate salt have displayed by far the greatest filaricidal activity of any of the group of more than sixty compounds tested by us. The chloride salt, when administered intraperitoneally to cotton rats at 8-hour intervals for 18 doses, was tolerated in a maximal total dose of 24 mg./kg. (1.33 mg./kg.  $\times$  18) and was curative in a minimal total dose of 0.3 mg./kg. (0.017 mg./kg.  $\times$  18), giving a therapeutic index of 80. When administered intraperitoneally once daily for 6 doses, the M.T.D. was 24 mg./kg. (total dose) and the M.C.D. was 0.3 mg./kg. (total dose), again giving a therapeutic index of 80. With subcutaneous administration every 8 hours for 18 doses, the M.T.D. was 72 mg./kg. and the M.C.D. 0.9 mg./kg., again giving a therapeutic index of 80. Administered subcutaneously once daily for 6 doses, the M.T.D. was 48 mg./kg. and the M.C.D. 0.3 mg./kg., giving a therapeutic index of 160. The *p*-toluene sulfonate salt of the same compound, when administered intraperitoneally every 8 hours for 18 doses, gave a M.T.D. of 30 mg./kg., a M.C.T. of 0.3 mg./kg. and a therapeutic index of 100. When administered intraperitoneally once daily for 6 days, the M.T.D. was 48 mg./kg., the M.C.D. 0.3 mg./kg., and the therapeutic index 160.

The acute intravenous L.D.<sub>50</sub> of the *p*-toluene sulfonate salt has been determined to be  $4.46 \pm 0.30$  mg./kg. for the rabbit,  $5.08 \pm 0.15$  mg./kg.

for the rat,  $5.36 \pm 0.27$  mg./kg. for the guinea pig, and  $5.89 \pm 0.23$  mg./kg. for the uninfected cotton rat. The acute lethal dose for dogs has been determined by administration to anesthetized dogs having the carotid artery cannulated for blood pressure recording. A dosage of 1 mg./kg. of the compound, as a 1:1000 solution, was injected intravenously at 15-minute intervals until the death of the animal. The lethal dose ranged from 8 to 26 mg./kg. with a mean of 12.3 mg./kg.

Studies of chronic toxicity in dogs are still in progress. Five dogs that received a dose of 1 mg./kg. intravenously daily (6 days per week) died or were sacrificed after 20 to 100 doses with an average of 37. These animals lost their appetite under daily injections and inanition appeared to play a major role as the cause of death. Dogs receiving a dose of 1 mg./kg. intravenously three times per week appear to tolerate the compound indefinitely and have received 75 to 100 injections without any gross evidence of injury.

## SUMMARY AND CONCLUSIONS

A group of more than sixty cyanine and related compounds have been tested for filaricidal activity in the natural *Litomosoides carinii* infection in the wild cotton rat.

The compounds tested were chiefly *p*-amino styryl quinolines and diquinolines, although other structures were investigated in an attempt to elucidate the relationship between structure and activity.

Filaricidal activity does not appear to depend upon the presence of any one structure, since it was found by us in *p*-amino styryl quinoline and pyridine derivatives, in diquinoline cyanines and carbocyanines, as well as in benzothiazole derivatives, and has been found by Welch and associates in pyrrol and pyrimidyl quinolines and other structures.

The essential structural grouping appears to be a quaternary and a tertiary nitrogen pair separated by an uneven number of carbon atoms containing alternate single and double bonds. Any interference with such a resonating system reduces or destroys filaricidal activity.

The cyanine derivatives that possessed the greatest degree of filaricidal activity in the cotton rat, out of the more than sixty such compounds tested by us, were 1,1'-di-*B*-ethoxyethyl, 2,2'-carbocyanine chloride and *p*-toluene sulfonate. These compounds were effective in curing this infection when administered intraperitoneally in dosages of 0.0167 mg./kg. every eight hours for 18 doses, or 0.05 mg./kg. once daily for six days. The therapeutic indices of the compounds (M.T.D./M.C.D.) ranged from 80 to 160.

The *p*-toluene sulfonate salt is ultimately toxic if administered intravenously to dogs daily in a dose of 1 mg./kg., the average lethal dose being 37 mg./kg., but is tolerated indefinitely (75-100 doses) when administered at the same dosage level three times weekly.

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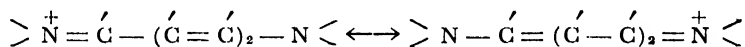
# EFFECT OF CYANINE DYES ON THE METABOLISM OF *LITOMOSOIDES CARINII*\*

By ERNEST BUEDING

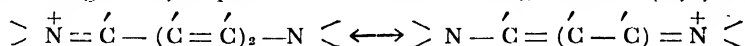
Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio

The cyanine dyes in concentrations as low as  $5 \times 10^{-8}$  M (1:40,000,000) inhibit the oxidative metabolism of *Litomosoides carinii*. The inhibition of the respiration of these worms produced by the cyanines is associated with a compensatory increase in aerobic glycolysis. This effect of the cyanines on filarial metabolism is not limited to conditions *in vitro*. After the intraperitoneal injection of subcurative doses of a cyanine dye to filariae infected cotton rats, the worms removed from the pleural cavity of the host survive and remain motile, but their oxygen uptake is decreased and their rate of aerobic glycolysis is increased. Since the administration of two to four times higher doses results in the death of the parasite, it appears that the curative action of the cyanines in filariasis of the cotton rat is due to an inhibitory effect of these compounds on the respiratory metabolism of the parasite.

The respiratory metabolism of *L. carinii* is inhibited by low concentrations of all compounds having the amidinium ion system in which a positively charged quaternary nitrogen is linked to a tertiary nitrogen by a conjugated chain of at least three carbons in length. Both nitrogens may be incorporated in heterocyclic rings (cyanine dyes),



or one nitrogen may be part of a side chain of a ring structure (styryl dyes),



Activity *in vitro* is not restricted to any particular ring and is maintained despite variations in structure. However, any modification which abolishes the possibility of amidinium ion resonance results in a disappearance of high activity *in vitro*.

The cyanines in concentrations 500 to 1000 times higher than those required to inhibit the respiration of *L. carinii* have no effect on the oxygen uptake of slices and homogenates of mammalian tissues or on the activity of cytochrome *c* or cytochrome oxidase. This indicates that the

\* The work described in this and the following paper (by Dr. L. Peters) was done, in part, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Western Reserve University (August 1, 1944–October 31, 1945); in part, under a contract between the Office of the Surgeon General, U. S. Army (November 1, 1945–December 31, 1946); and in part with the aid of a grant from the U. S. Public Health Service (since January 1, 1947). From the beginning of the study of this group of compounds, we have enjoyed the finest cooperation from Dr. L. G. S. Brooker and from Parke, Davis Laboratories. Further, this investigation has been facilitated in innumerable ways by Lucille Farquhar, technical aide of the National Research Council, who coordinated studies in this and related fields. Other members of this series of compounds have been studied by R. N. Bieter, H. N. Wright, and their associates at the University of Minnesota.

cyanines inhibit in these filarial worms an enzyme system that plays no role, or one of only minor importance, in mammalian tissues. Since the respiration of this organism is markedly inhibited by a low concentration of cyanide, it appears likely that *L. carinii* has one or several heavy metal-containing respiratory systems. The latter are not, however, identical with cytochrome *c* or cytochrome oxidase, since neither of these two enzymes could be detected in this parasite. Respiration and carbohydrate utilization of *L. carinii* is inhibited by *p*-chloro-mercuric benzoate. The effect of this sulphhydryl group inhibitor is not reversed by cysteine, thioglycollate or glutathione.

Several metabolic characteristics, as, for instance, a high rate of aerobic glycogen synthesis, the quantitative conversion of carbohydrate to lactic acid, and the absence of a post-anaerobic increase in oxygen uptake, distinguish the filarial worm, *L. carinii*, from most of the other parasites which have been studied so far. This is only one example which illustrates the fact that, in contrast to vertebrates, the metabolic characteristics of invertebrates vary greatly from one species to another. Even among morphologically closely related species profound metabolic differences have been observed. For example, the sole end product of the aerobic carbohydrate metabolism of *Trypanosoma equiperdium* is pyruvic acid,<sup>1</sup> while *Trypanosoma lewisi* converts glucose aerobically to succinic, acetic and formic acids, ethyl alcohol and carbon dioxide.<sup>1</sup> Furthermore, respiration of *Trypanosoma cruzi* is inhibited in low concentrations of cyanide;<sup>2</sup> conversely, respiration of *Trypanosoma equiperdium* and of *Trypanosoma rhodesiense* is not inhibited by cyanide.<sup>3</sup>

Therefore, it is evident that the metabolic characteristics of one parasitic invertebrate do not permit one to conclude that a morphologically related organism has a similar type of metabolism and, consequently, is sensitive to the same metabolic inhibitors or chemotherapeutic agents. On the other hand, investigations of the biochemical characteristics of an invertebrate parasite will contribute to a better understanding of the mechanism of action of known chemotherapeutic agents effective against this specific organism. Furthermore, such an approach will eventually afford a possibility to replace the presently prevailing empirical methods with a more rational development of metabolic inhibitors and of chemotherapeutic agents against pathogenic parasites.

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# ANTIFILARIAL ACTION, TOXICOLOGY, AND CLINICAL TRIAL OF CYANINE DYES IN FILARIASIS\*

By LAWRENCE PETERS

*Department of Pharmacology, School of Medicine, Western Reserve University,  
Cleveland, Ohio*

Routine testing of many compounds for chemotherapeutic properties against *L. carinii*, in the cotton rat, disclosed that the cyanine dye (1-amy-2,5-dimethyl-3-pyrrole) (1,6-dimethyl-2-quinoline) dimethincyanine chloride (Chemotherapy Center #348), when administered intraperitoneally every 24 hours for 5 doses (0.2 mg./kg. per dose), consistently killed all worms present in the pleural cavity as indicated by the irreversible loss of motility of such worms when placed in nutrient medium 48 hours after the last dose. Maximally tolerated doses were of 10 to 12 times this magnitude. No decrease in microfilaremia occurred during this period, but when autopsy was delayed, a slow reduction in the microfilarial count occurred over a period of six months. In contrast, it was not possible to produce a lethal effect against the adult forms of the heart-worm, *Dirofilaria immitis*, in dogs, but a marked reduction in the microfilarial count occurred during a period of only a few days following even a single dose of drug.

The inhibitory effects of cyanines on the essential oxidative metabolism of *Litomosoides carinii*, and the chemical structure essential to those effects, have been discussed by Dr. Bueding in the preceding paper.

"Screening" tests on the large number of cyanines prepared by Dr. L. G. S. Brooker consisted of measurements of curative activity and toxicity in cotton rats, and measurements of ability to inhibit the oxidative metabolism of *L. carinii* *in vitro*. On the basis of such tests, 1-ethyl-3,6-dimethyl-2-phenyl-4-pyrimido-2-cyanine chloride (C.C. #863) was selected as the most promising compound, its curative activity *in vivo* being equal to, and its activity *in vitro* superior to, that of compound #348.

Given orally, the cyanines produced only occasional cures even with massive doses. Subcutaneous therapy likewise produced cures inconsistently, even at maximally tolerated doses, and was accompanied by tissue damage at the site of injection. Though tissue damage was less with compound #863 than with other cyanines, it was sufficient to preclude the subcutaneous route of administration. Therefore, compound #863 was investigated from the standpoint of intravenous therapy.

\* We are indebted to Dr. W. B. Wartman, Department of Pathology, Northwestern University Medical School, Chicago, Illinois, for his cooperation in the pathological examination of the tissues of animals subjected to treatment with #863.

In infected cotton rats, cures were almost invariably produced by 1.0 mg./kg., repeated 3-6 times at intervals of 1, 3 or 7 days, while fatalities occurred only with individual doses of 10 mg. or more per kg.

Extensive studies of the chronic toxicity of compound #863 in dogs and monkeys disclosed a mild and reversible renal damage as the only manifestation. During intravenous administration, a transient hypotensive effect with compensatory tachycardia was noted. This effect is of consequence only when large doses are administered rapidly.

A method of extraction of the drug from tissues and its spectrophotometric determination have been devised. Data on distribution and excretion of drug, following its administration in dogs, will be presented and discussed. These data show that the kidney, and particularly the renal tubules, took up an amount of drug much greater than that found in any other tissue or organ. Though the heart contained very low concentrations of drug following a single dose, cumulation tended to occur in this organ and in skeletal muscle with multiple doses given at intervals of 24 hours. The concentrations of drug found in other organs were not striking. Excretion of drug by the bile in amounts up to 15 per cent of an injected dose may explain the absence of high concentrations in the liver. Measurements of the recovery of drug from organs, bile, and urine suggest that degradation in the body occurred to such an extent that 2.0 mg./kg. could be given to dogs daily with only slight cumulation. Marked cumulation occurred, however, when the dose was increased to 5.0 mg./kg.

Studies on the possible effectiveness of compound #863 in the treatment of human patients infested with *Wuchereria bancrofti* have been initiated in Puerto Rico, through the courtesy of the staff of the School of Tropical Medicine in San Juan. Occasional nausea and a mild transient fall in blood pressure with compensatory tachycardia were the only untoward reactions observed in the 27 patients treated. The therapy consisted of 4 or 8 intravenous infusions of 2.0 mg./kg. of drug with a 48-hour interval between doses. Syringe injections of 1.0 mg./kg., administered twice weekly for 4 weeks, were likewise well tolerated. Microfilaria counts performed on the peripheral blood of these patients showed a marked reduction of the microfilaremia during and immediately following therapy. However, within a few weeks, the number of circulating microfilariae had returned to pre-treatment levels and has remained thus for a period of 9 to 10 months.

More recently, ten additional patients have been treated with daily intravenous infusions of 2.0 mg./kg. of drug. In these cases, signs of toxicity, including dyspnea, severe persistent vomiting, headache, and a marked hypotension with bradycardia, began to make their appearance after the second or third dose. As a result, therapy was discontinued in three cases and temporarily interrupted in the remainder, who received two additional doses after a 48-hour rest period. As in the previous study, a marked but transient reduction in microfilaria counts occurred with a return to pre-treatment levels 1 to 4 weeks later.

The second series of patients will have to be followed for some months before the effectiveness of the therapy can be finally appraised. However, in the case of the first series of patients, the failure of the therapy to produce a permanent effect on the microfilaremia strongly suggests that no adult worms were killed.

In attempting to explain this lack of a chemotherapeutic effect against *W. bancrofti*, the possibility that compound #863 was incapable of penetrating the lymphatic system presented itself. However, lymph collected from the thoracic duct of dogs following intravenous administration of this drug was found to be capable of inhibiting the respiration of adult *L. carinii* *in vitro*. Therefore, a more likely explanation for the apparent therapeutic failure of the drug against *W. bancrofti* is the existence of a difference in the metabolic characteristics of this parasite from those of *L. carinii*.

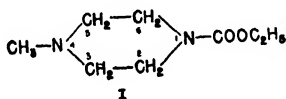


# THE CHEMISTRY OF PIPERAZINE COMPOUNDS IN THE CHEMOTHERAPY OF FILARIASIS

By S. KUSHNER, L. M. BRANCONE, R. I. HEWITT,  
W. L. MCEWEN, AND Y. SUBBAROW  
*Lederle Laboratories Division, American Cyanamid Company,  
Pearl River, New York*

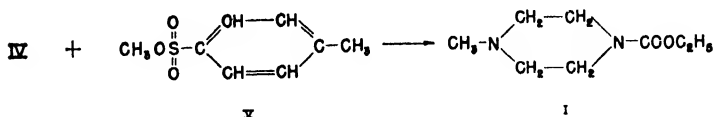
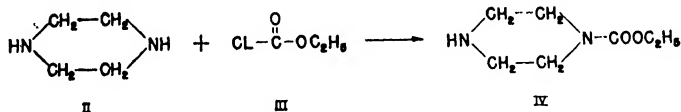
and H. W. STEWART, R. J. TURNER, AND J. J. DENTON  
*Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey*

For some time, our laboratory has been actively interested in obtaining an active filaricide that would be administered orally and still be non-metallic and thus avoid the usual effects of the heavy metals in body assimilation. Screening experiments against microfilariae in the cotton rat led us to believe that we might achieve our goal in the piperazine nucleus.



The first compound that showed great promise in the initial screening was 1-carbethoxy-4-methylpiperazine (I).

This compound, designated as 180-C, was prepared by reacting ethyl chlorocarbonate (III) under controlled conditions with piperazine (II) in aqueous solution. The resulting 1-carbethoxypiperazine (IV), which



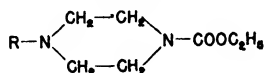
is obtained in good yield, can be reacted with methyl tosylate (V) in alcoholic solution to give 180-C. This method was used by Moore and co-workers<sup>1</sup> in the preparation of the ethyl analogue.

Subsequent variations of this molecule have led to preparation of more than 60 compounds for chemotherapeutic testing.

In our initial experimentation, the carbethoxy group on the 1 position was kept constant while different groups were placed in the 4 position.

In TABLE 1, these compounds are listed with a few of their physical properties and their relative activity in the cotton rat. The first eight compounds listed in this table were prepared in the same manner as designated for 180-C, with the appropriate changes in the alkyl tosylate for compounds 3, 4, 5, and 6.

TABLE 1



Compound	R	B.P. of base °C.	Mm.	M.P. of HCl °C.	Activity
1	H			156.5-157	+
2	CH <sub>3</sub>	97-98	8	168.5-169	++
3	C <sub>2</sub> H <sub>5</sub>	132	28		+
4	<i>n</i> -C <sub>3</sub> H <sub>7</sub>	136	16	189-192	+
5	<i>iso</i> -C <sub>3</sub> H <sub>7</sub>	138-144	19		+
6	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	139-140	8		+
7	<i>sec.</i> -C <sub>4</sub> H <sub>9</sub>	139-147	18	218-221 (dec:)	+
8	<i>iso</i> -C <sub>4</sub> H <sub>9</sub>	139-143	18		—
9	<i>n</i> -C <sub>7</sub> H <sub>15</sub>	159-161	4		—
10	CH <sub>2</sub> =CH CH <sub>3</sub>	113-115	6		—
11	CH <sub>2</sub> CH <sub>2</sub> OH	175-177	12		—
12	CH <sub>2</sub> CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub>	123-125	2		—
13	C <sub>6</sub> H <sub>5</sub>	61-61.5 (m.p.)		197-198 (dec:)	—
14	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub>			218-218.5	—
15	CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub>	131-133	3		—

The *n*-heptyl, allyl,  $\beta$ -hydroxy ethyl, benzyl, and carbethoxymethyl derivatives were prepared by the use of their corresponding halide in alcoholic solution with 1-carbethoxypiperazine.

The dicarbethoxypiperazine was prepared by the action in neutral or alkaline solution with piperazine and ethyl chlorocarbonate. It is usually isolated as the by-product in the preparation of 1-carbethoxypiperazine, the starting material for most of the compounds in TABLE 1.

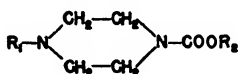
The 1-carbethoxy-4-phenylpiperazine was prepared by direct reaction of ethyl chlorocarbonate on 1-phenylpiperazine.

No gain in activity was shown by homologous preparation; rather, activity decreases with the increasing radical size. Substitution of such solubilizing groups as the  $\beta$ -hydroxyethyl showed no advantages.

Various compounds similar to 180-C were made, wherein the carbethoxy group was replaced by lower or higher carbalkoxy homologues. These

were prepared in the same manner as that outlined for 180-C, with the corresponding substitution for the alkyl chlorocarbonate. These compounds, tabulated in TABLE 2, show no enhanced activity but rather a decrease in activity.

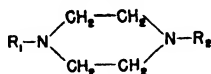
TABLE 2



Compound	R <sub>1</sub>	R <sub>2</sub>	B.P. of base °C.	Mm.	Activity
1	H	CH <sub>3</sub>	112-116	7	—
2	CH <sub>3</sub>	CH <sub>3</sub>	116-121 (m.p.)	—	—
3	CO <sub>2</sub> CH <sub>3</sub>	CH <sub>3</sub>	163	11	—
4	H	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	141-143	10	—
5	CO <sub>2</sub> C <sub>4</sub> H <sub>9</sub> - <i>n</i>	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	205-208	10	—
6	H	<i>iso</i> -C <sub>4</sub> H <sub>9</sub>	138-142	13	— —
7	CO <sub>2</sub> C <sub>4</sub> H <sub>9</sub> - <i>iso</i>	<i>iso</i> -C <sub>4</sub> H <sub>9</sub>	203-205	15	—

Certain 1-alkylpiperazines have been made by Baltzly<sup>2</sup> by the alkylation of 1-benzylpiperazine in the 4 position with subsequent removal of the benzyl group by catalytic hydrogenation. A more indirect procedure has been used by Prelog and Stepan<sup>3</sup> for the preparation of 1-methylpiperazine from 1-methyl-4-phenylpiperazine. Moore, Boyle, and Thorn<sup>1</sup> obtained 1-ethylpiperazine from the hydrolysis of 1-carbethoxy-4-ethylpiperazine in concentrated hydrochloric acid. We have used this latter method for preparing the 1-alkylpiperazines reported in TABLE 3.

TABLE 3



Compound	R <sub>1</sub>	R <sub>2</sub>	B.P. of base °C.	Mm.	M.P. of base HCl °C.	Activity
1	CH <sub>3</sub>	H	134-136	760	82.5-83	—
2	CH <sub>3</sub>	CH <sub>3</sub>	131-133	760	251.5-253 (dec.)	—
3	CH <sub>3</sub>	CH <sub>2</sub> CH <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	—	—	262-264	—
4	C <sub>2</sub> H <sub>5</sub>	H	155-158	760	—	—
5	<i>iso</i> -C <sub>3</sub> H <sub>7</sub>	H	—	—	274-275 (dec.)	—
6	C <sub>6</sub> H <sub>5</sub>	H	161-164	—	245-247	+
7	C <sub>6</sub> H <sub>5</sub>	CH <sub>3</sub>	130-131	—	180-182 (dec.)	—

The 1,4 dimethylpiperazine was prepared by the reaction of piperazine with formaldehyde and formic acid. This mode of methylation was also used for large-scale preparation of 180-C.

The 1-methyl-4- $\beta$ -dimethylaminoethyl piperazine was prepared by the interaction of methylpiperazine and  $\beta$ -dimethylaminoethyl chloride. None of these compounds showed appreciable activity.

TABLE 4

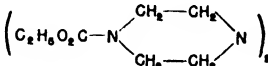
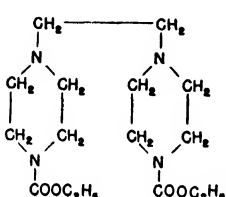
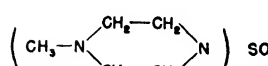
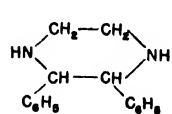
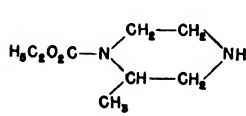
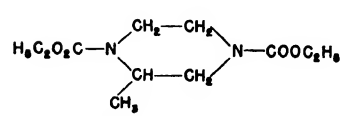
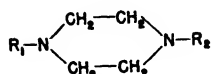
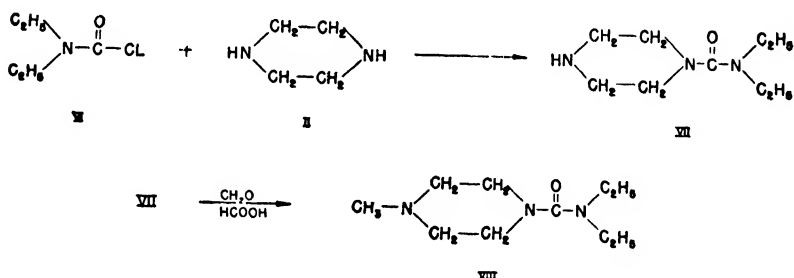
Compound	Miscellaneous	B.P. of base °C.	Mm.	M.P. of base HCl °C.	Activity
1		CH <sub>2</sub>	61.5-62.5 (m.p.)		+
2			80-80.5		—
3		SO <sub>2</sub>	89-90.5 (m.p.)		—
4				310-311	—
5			127-129	18	—
6			173-175	16	+

TABLE 7



Compound	R <sub>1</sub>	R <sub>2</sub>	B.P. of base °C.	Mm.	M.P. of HCl °C.	Activity
1	CH <sub>3</sub> -	-CON(CH <sub>3</sub> ) <sub>2</sub>			180-181	++
2	CH <sub>3</sub> -	-CON C <sub>2</sub> H <sub>5</sub>			177 (dec:)	+
3	CH <sub>3</sub> -	H -CON(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	108.5-111	3	156.5-157	++
4	CH <sub>3</sub>	-CON CH(CH <sub>3</sub> ) <sub>2</sub>			200-203	+
5	CH <sub>3</sub>	-CON(η-C <sub>4</sub> H <sub>9</sub> ) <sub>2</sub>			151-152	—
6	CH <sub>3</sub>	-CON-C <sub>6</sub> H <sub>5</sub>			126-130	—
7	CH <sub>3</sub>	H -CON-CH <sub>2</sub> -C <sub>6</sub> H <sub>5</sub>			188-192	—
8	CH <sub>3</sub>	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\  \diagup \quad \diagdown \\  -\text{C}-\text{N} \quad \text{N}-\text{CH}_3 \\  \parallel \quad \diagdown \quad \diagup \\  \text{O} \quad \text{CH}_2-\text{CH}_2  \end{array}  $			303-304	—
9	CH <sub>3</sub>	$  \begin{array}{c}  \text{CH}_2-\text{CH}_2 \\  \diagup \quad \diagdown \\  -\text{C}-\text{N} \quad \text{O} \\  \parallel \quad \diagdown \quad \diagup \\  \text{O} \quad \text{CH}_2-\text{CH}_2  \end{array}  $	178-179	17	52-55**	+
10	i-C <sub>3</sub> H <sub>7</sub> -	-CON(C <sub>3</sub> H <sub>7</sub> ) <sub>2</sub>		16	206-208.5	—

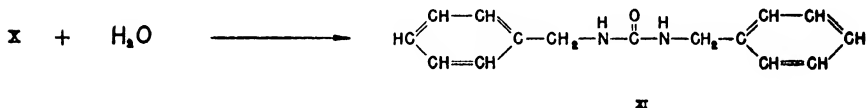
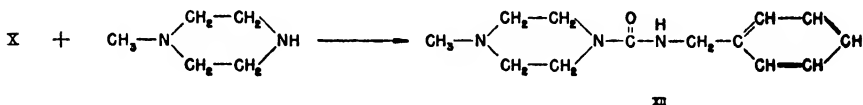
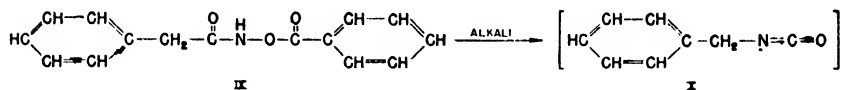
\*\* M.p. of free base.



Compounds 2 and 6 were prepared by the direct reaction of the corresponding isocyanate with 1-methylpiperazine.

The synthesis of 1-benzylcarbonyl-4-methylpiperazine was effected by a modification of the procedure of James<sup>6</sup> wherein the benzoate of phenyl acethydroxamic acid (IX), when heated with aqueous alkali, gives symmetrical dibenzylurea (XI) presumably through the intermediate benzyl isocyanate (X). Instead of heating the benzoate with alkali,

2 moles of 1-methylpiperazine are added; 1 mole causes the intermediate formation of the isocyanate, which immediately reacts with the remaining 1-methylpiperazine to give the desired 1-benzylcarbamyl-4-methylpiperazine (XII).



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# PARASITOLOGY OF PIPERAZINES IN THE TREATMENT OF FILARIASIS

By R. I. HEWITT, D. E. WHITE, S. KUSHNER, W. S. WALLACE,  
H. W. STEWART, AND Y. SUBBAROW

*Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.,  
and Calco Chemical Division, American Cyanamid Company, Bound Brook, N. J.*

Previous studies on the filaricidal properties of various drugs in naturally acquired infections with *Litomosoides carinii* in cotton rats,<sup>1-5</sup> *Dirofilaria immitis* in dogs,<sup>6-7</sup> and *Wuchereria bancrofti* in human patients<sup>8, 9</sup> have been confined largely to organometallic compounds. With the exception of certain cyanine dyes,<sup>4</sup> no chemical compound not containing antimony or arsenic has been shown to produce marked filaricidal activity in any of the above hosts. Moreover, the above classes of compounds produce their maximum effects when administered parenterally.

During the course of a screening program for filaricides in these laboratories, using cotton rats naturally infected with *Litomosoides carinii* as test animals, marked activity was found with a number of piperazine compounds. The most active members of the series studied produce filaricidal effects in cotton rats quite different from any other known group of compounds, in that (1) an immediate reduction in microfilariae occurs following the first dose, (2) the adult worms are killed slowly, after prolonged treatment, and (3) oral administration is equally as effective as parenteral administration. Subsequent investigations showed that these effects could be carried over to *Dirofilaria*-infected dogs, and to human patients infected with *Wuchereria bancrofti*.

The results obtained in cotton rats and dogs with various piperazines, and in particular with 1-diethylcarbamyl-4-methylpiperazine hydrochloride (Hetrazan), form the subject of this paper. The general procedures used for studying the action of chemical compounds in cotton rats and dogs have been discussed previously.<sup>10</sup>

**Types of Piperazines Studied.** The first piperazine compound found to produce a measurable effect against *Litomosoides carinii* was 1-carbethoxy-4-methylpiperazine hydrochloride. In intraperitoneal doses ranging from 6.25 to 200 mg. per kg. twice daily, this compound produced marked reductions in circulating microfilariae (TABLE 1). Oral doses within the same range were found to be equally effective against microfilariae. Reductions as high as 95 per cent occurred in some animals on the first day after treatment was initiated, and were sustained as long as treatment was continued. Very little effect with this compound could be demonstrated against adult worms, however, particularly in early stages of the investigation, and a number of related compounds were therefore prepared by our organic chemistry division in order to determine whether the activity could be enhanced.

The piperazine nucleus itself showed no activity, nor did any of its components. A carbethoxy radical in position "1", with various substitu-

tions in position "4" produced a number of compounds with high micro-filaricidal activity (TABLE 2), and some which showed a suggestive effect against adult worms. As the alkyl chain was increased, however, the toxicity became greater and higher doses were necessary to produce measurable effects.

The only compounds lacking the carbethoxy group which showed marked activity against microfilariae were 1-ethylcarbamy-4-methylpiperazine hydrochloride (182-L); 1-diisopropylcarbamy-4-methylpiperazine hydrochloride (177-L); 1-dimethylcarbamy-4-methylpiperazine hydrochloride (152-L); and 1-diethylcarbamy-4-methylpiperazine hydrochloride (84-L). The latter two compounds are considered as the most active of the piperazines yet tested.

A relatively large number of piperazines (approximately 21 per cent) of the 126 tested thus far have shown measurable effects against the microfilariae. An exhaustive study of all active members of the group

TABLE 1  
MICROFILARICIDAL EFFECT OF FOUR PIPERAZINE COMPOUNDS IN  
COMMON RATS

No.	Name	Dose intra- perito- neal mg./kg. b.i.d.	Microfilariae per 100 fields days during treatment						
			1	2	3	4	5	6	7
162-C	1-Carbethoxy- piperazine Hydrochloride	25	180	6	4	6	6	8	6
		50	652	252	96	18	14	14	12
		100	1,360	84	8	10	4	6	6
180-C	1-Carbethoxy-4- methylpiperazine Hydrochloride	6.25	430	190	56	250	52	8	22
		12.5	128	72	60	48	22	12	56
		25	160	112	28	38		6	14
		50	384	40	22	12	4	0	4
		100	264	26	8	2	6	8	4
		200	168	12	2	1	1	4	0
61-L	1-Guanyl-4- carbethoxy-pi- perazine Hydrochloride	25	380	212		436	272	412	304
		50	388	68		18	4	24	6
		100	104	12		6	8	6	8
84-L	1-Diethylcarbamy- 4-methylpiperazine Hydrochloride	1.5	480	400	340	270	290	210	184
		3.13	1,220	108	40	56	4	8	2
		6.25	600	116	44	22	72	40	22
		12.5	180	64	8	8	8	1	8
		25	396	100	20	8	10	6	1
		50	476	76	22	13	12	2	3
		100	192	12	6	5	4	10	1
	Non-treated con- trols		1,630	252	590	1,560	224	1,020	1,180
			46	20	28	18	44	40	72
			36	26	56	44	52	58	36
			40	60	88	22	40	24	56
			560	212	208	184	280	244	410



TABLE 2

COMPARATIVE MICROFILARICIDAL ACTIVITY AND TOXICITY OF 18 PIPERAZINE DERIVATIVES IN COTTON RATS, USING 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HYDROCHLORIDE (HETRAZAN) AS A STANDARD

No.	Name	Minimum effective dose against microfilariae mg./kg. intraperitoneal	LD <sub>50</sub> Mice mg./kg. intraperitoneal	Approximate Hetrazan equivalent	Approximate chemotherapeutic index
163-C	1-Phenylpiperazine hydrochloride	50	140	0.06	2.8
162-C	1-Carbethoxypiperazine hydrochloride	25	275	0.125	11.0
217-L	1 Methyl-4-(4'-morpholine carbamyl)-piperazine hydrochloride	12.5	?	0.25	?
180-C	1-Carbethoxy-4-methylpiperazine hydrochloride	6.25	550	0.5	88.0
59-L	1-Carbethoxy-4-ethylpiperazine	50	175	0.06	3.5
82-L	1-Carbethoxymethylpiperazine	100	?	0.03	?
U-653	1-Carbethoxy-4-propylpiperazine hydrochloride	25	87.5	0.125	3.5
147-L	1-Carbethoxy-4-isopropylpiperazine hydrochloride	25	100	0.125	4.0
U-655	1-Carbethoxy-4-butylpiperazine	50	125	0.06	2.5
U-801	1-Carbethoxy-4- <i>s</i> butylpiperazine	100 Oral	175	0.05	1.75*
61-L	1-Guanyl-4-carbethoxypiperazine sulfate	50	285	0.06	5.6
218-C	<i>Bis</i> -(4-carbethoxy-1-piperazine)-methane	25	175	0.125	7.0
169-C	1,4-Dicarbethoxypiperazine	100	500	0.03	5.0
76-L	1,4-Dicarbethoxy-2-methylpiperazine	200 Oral	375	0.025	1.9
182-L	1-Ethylcarbamyl-4-methylpiperazine hydrochloride	6.25	2,250	0.5	360.0
152-L	1-Dimethylcarbamyl-4-methylpiperazine hydrochloride	6.25	310	0.5 to 1.0	48.0
177-L	1-Diisopropylcarbamyl-4-methylpiperazine hydrochloride	6.25	160	0.5	25.6
84-L	1-Diethylcarbamyl-4-methylpiperazine hydrochloride	3.13	285	1.0	91.0

\* LD<sub>50</sub> determined by intraperitoneal injections, and minimum effective dose by oral administration.

has not been made. The selection of Hetrazan as the most promising of the derivatives made available was based upon the repeated marked effects obtained against microfilariae in low doses, lethal action against adult worms after continued dosage, and comparatively low toxicity.

**Effect of Piperazines on Circulating Microfilariae in Cotton Rats.**

The first measurable effect produced by some piperazine compounds in filaria-infected cotton rats is the immediate and precipitous reduction in microfilariae. As illustrated in TABLE 3, a large proportion of the microfilariae disappears after a single dose of 5, 10 or 25 mg. per kg. of Hetrazan, and similar effects are produced with related compounds. Oral treatment is as effective in rapidly reducing the microfilariae as is parenteral treatment (TABLE 4).

The amount of drug necessary to produce an immediate microfilaricidal effect varies with different piperazines. As demonstrated in TABLE 1, 50 mg. per kg. is necessary in the case of 1-guanyl-4-carbethoxypiperazine hydrochloride, whereas 3 mg. per kg. of Hetrazan will invariably produce a sharp reduction in microfilariae. This measurable difference in dosage response provides a quick, qualitative index of activity (TABLE 2) and has been used in the selection of the most active members of the series.

During treatment with active piperazines, the microfilariae continue to remain at a very low level or disappear from the peripheral blood completely (TABLES 5, 6, and 7). Following cessation of treatment, they may recur quickly, slowly, or not at all (TABLE 7). The extent of recurrence is related somewhat to the amount and frequency of dosage, although no predictions can be made in this respect. No definite relationship exists between the recurrence of microfilariae and the height of the initial count (TABLE 7).

TABLE 3

RESULTS OF TREATMENT IN COTTON RATS WITH HETRAZAN EVERY 2 HOURS FOR 48 HOURS

Rat No.	Dose mg. kg.	Microfilariae per 100 fields						Dead worms at autopsy 14th day			
		Before treatment	2 hrs.	12 hrs.	24 hrs.	6 days	13 days	100%	50 to 95%	Less than 50%	None dead
1292	25	248	22	2	0	2	6		X		
1278	25	156	3	0	0	4	16			X	
1253	25	320	22	0	0	0	16				X
1297	25	964	24	0	0	0	18		X		
1259	25	664	40	3	1	6	30			X	
1266	10	676	8	8	2	4	12			X	
1264	10	248	8	0	0	0	16			X	
1267	10	748	52	14	1	2	48		X		
1265	10	604	22	16	4	9	92			X	
1281	5	192	8	0	0	0	6				X
1271	5	88	48	6	0	6	26				X
1299	5	324	52	3	0	3	36				X
1258	5	380	204	14	6	4	38				X
1279	5	176	16	2	0	1	46				X
1291	5	276	4	20	0	6	84				X

TABLE 4

COMPARISON OF THE MICROFILARICIDAL EFFECT OF INTRAPERITONEAL AND ORAL  
DOSES OF HETRAZAN IN COTTON RATS

Rat No.	Dose mg./kg. b.i.d.	Microfilariae per 100 fields, days during treatment							
		1	2	3	4	5	6	7	14
566	5 I	92	10	2	2	4	4	6	4
579	5 I	36	14	1	1	1	0	0	4
571	5 I	76	8	14	24	22	8	1	1
900	5 O	48	12	4	5	1	0	1	0
934	5 O	228	14	0	10	3	2	4	2
897	5 O	248	26	22	4	8	3	8	8
565	10 I	112	10	6	2	0	0	0	0
583	10 I	100	52	8	5	22	10	8	6
588	10 I	232	56	2	6	1	0	0	0
927	10 O	44	20	14	6	6	0	2	2
908	10 O	408	10	6	10	3	2	1	0
909	10 O	56	14	8	6	1	0	1	1
567	25 I	364	20	1	3	1	2	2	1
572	25 I	692	20	0	0	4	4	0	1
587	25 I	452	8	1	2	2	0	1	1
895	25 O	72	3	0	0	0	0	0	0
940	25 O	168	8	0	0	0	0	0	0
913	25 O	40	1	2	0	0	1	0	0

I = Intraperitoneal; O = Oral.

TABLE 5

COTTON RATS TREATED WITH HETRAZAN IN WHICH LOW EMBRYO COUNTS AT THE  
TIME OF AUTOPSY WERE ASSOCIATED WITH THE DEATH OF THE MAJORITY OF  
ADULT WORMS

Rat No.	Dose mg. kg.*	No. days treated	Microfilariae per 100 fields, days										Day of au- topsy
			1	7	14	21	28	35	42	49	58	107	
488	3 I	16	132	1	1	0	0	1	0				43rd
629	3 I	30	60	0	0	0	0						31st
617	25 I	28	108	0	0	0	1	0	8	1	0		58th
599	25 I	28	64	0	0	0	0	0	4	1	1		58th
593	25 I	30	22	0	0	0	0	2	1				44th
624	25 I	30	88	0	2	1	0	3	4				44th
1050	25 I	30	72	0	0	0	0		6				52nd
1074	25 I	30	124	0	0	0	0		0				52nd
547	25 O	30	26	0	0	0	0						33rd
925	25 O	14	36	0	0	6	20	0	1				42nd
895	25 O	14	72	0	0	0	0	0	0				42nd
940	25 O	14	168	0	0	14	8	0	0				42nd
637	25 O	14	156	0	0	1	0	0	0				42nd
1325	25 O	30	140		0							0	107th
1368	25 O	30	56		0							0	107th
1453	25 O	30	36		0							3	107th
634	50 O	28	56	0	0	0	0	1	4	8	8		58th
552	100 I	30	36	0	0	0	0						33rd

\* Two or three times daily.

I = Intraperitoneal; O = Oral.

TABLE 6

COTTON RATS TREATED WITH HETRAZAN IN WHICH LOW EMBRYO COUNTS AT THE TIME OF AUTOPSY WERE ASSOCIATED WITH THE ABSENCE OF ADULT WORMS

Rat No.	Dose mg. kg.*	No. days treated	Microfilariae per 100 fields, days										Day of autopsy
			1	7	14	21	28	35	42	49	58	107	
481	3 I	16	68	0	2	1	4	4					43rd
543	3 I	32	128	3	0	0	0	0	0	0			70th
1402	25 O	30	330		0							0	107th
1406	25 O	30	820		0							0	107th
1409	25 O	30	130		0							0	107th
1418	25 O	30	124		0							0	107th
1425	25 O	30	84		0							0	107th
1511	25 O	30	160		0							0	107th
1540	25 O	30	145		1							0	107th
1544	25 O	30	86		0							6	107th
1533	25 O	30	96		0							0	107th
1403	25 O	30	28		0							0	107th
1421	25 O	30	210		0							0	107th
1414	25 O	30	14		0							0	107th
611	50 O	28	44	0	0	0	0	0	0	0	0		58th
529	50 O	30	16	0	1	0	0						33rd
623	50 O	28	52	0	0	0	0						30th

\* Two or three times daily.

I = Intraperitoneal; O = Oral.

TABLE 7

COTTON RATS TREATED WITH HETRAZAN IN WHICH LOW EMBRYO COUNTS AT THE TIME OF AUTOPSY WERE ASSOCIATED WITH THE PRESENCE OF LIVING ADULT WORMS

Rat No.	Dose mg. kg.*	No. days treated	Microfilariae per 100 fields, days									Day of autopsy
			1	7	14	21	28	35	42	49	58	
484	3 I	16	224	8	6	7	0	0				36th
486	3 I	16	236	2	0	0	1	0				36th
487	3 I	16	204	6	4	16	24	64			0	58th
480	6¼ O	15	46	10	1	1	2	0		0		49th
908	10 O	14	408	1	0	6	24	10	8			42nd
917	10 O	14	1,260	0	1	0	6	1	0			42nd
548	25 I	30	184	0	0	0	0	0				35th
921	25 O	14	96	1	0	2	0	2	10			42nd
775	25 O	14	224	1	0	1	4	0	4			42nd
1051	25 O	30	96	1	0	0	0	0		0		52nd
621	50 O	28	608	0	0	0	0	0	0			42nd
555	50 O	30	100	0	1	0	0	0				35th

\* Two or three times daily.

I = Intraperitoneal; O = Oral.

It will be shown, later, that Hetrazan produces a slow lethal effect against the adult worms in cotton rats. If all adult worms are killed or are sterile at the end of treatment, and the microfilariae have all been removed, recurrences in microfilariae following cessation of treatment will

certainly not occur. The presence of some living adult worms, or of worms which have not been totally affected by treatment with Hetrazan or other piperazine compounds, will be revealed in most cases by recurrences in microfilariæ when treatment is stopped.

The mode of action of piperazines against microfilariæ is not known. Rat and frog microfilariæ placed in high dilutions of several members of the series (1-1,000, 1-10,000) undergo contortion and spasmodic contraction. The microfilariæ of *Folyella dolichoptera* from Southern frogs<sup>11</sup> demonstrate this effect better than those of *Litomosoides*. These are very long embryos with a narrow whip-like anterior end and a thicker posterior end. They contract immediately into a tight coil when placed in contact with piperazine solutions, and the same effect can be observed in the blood of frogs shortly after treatment has been administered. This observation, plus the fact that adult *Litomosoides* removed from rats in early stages of treatment show no demonstrable damage to the embryos *in utero*, seems to suggest that the piperazines act directly upon the microfilariæ in the peripheral blood.

**Effect of Hetrazan Upon Adult Worms in Cotton Rats.** It was discovered early during the course of these investigations that the effect of piperazine compounds against adult worms in cotton rats at any given dosage was not predictable. Several of the compounds shown in TABLE 2 killed some adult worms following various dosage regimes, but none were uniformly effective. It was decided, therefore, to choose the most effective derivative against microfilariæ, and one with accompanying low toxicity, to test extensively against this infection, particularly for activity against the adult worms. Hetrazan (1-diethylcarbamyl-4-methylpiperazine hydrochloride) appeared to possess the greatest microfilaricidal activity of the derivatives then available, and was selected for comprehensive study.

Among the variables considered were the amount of drug given, frequency of administration, route of administration, height of the initial microfilaria count, and number of days elapsed from cessation of treatment to autopsy. It was found very soon that the route of administration, whether oral or intraperitoneal, made no difference in the results.

Various dosage regimes were then administered, and in no case were the effects upon the adult worms definitely predictable, even though the majority of circulating microfilariæ were quickly removed in each instance. As demonstrated in TABLES 5, 6, and 7, following treatment with various doses, some of the rats revealed the majority of worms dead or no worms whatever at autopsy, while in others all of the worms were living, even though recurrences in microfilariæ to any large degree did not occur after cessation of treatment. In other cases (TABLES 8 and 9), varying percentages of adult worms were found at autopsy and recurrences in microfilariæ following treatment occurred.

The frequency of treatment, within certain limits, did not materially change the above effects upon adult worms. For example, rats treated every two hours for forty-eight hours (TABLE 3), although exhibiting very

rapid clearance of circulating microfilariae, did not reveal all dead adult worms at autopsy.

The amount of drug given in a single dose has varied from 1.5 mg. per kg. to 200 mg. per kg. In general, doses of 10 mg. per kg. or higher produced more consistent results against adult worms than lower doses, although good effects were consistently obtained against microfilaria at 3 mg. per kg.

Attention was then directed to the length of time elapsing between cessation of treatment and autopsy. In one series, as indicated in TABLE 8, rats receiving the same dosage revealed more dead worms when autopsied four weeks after cessation of treatment than when autopsied immediately after treatment. Accordingly, protocols were devised in an attempt to determine the ratio between optional periods of treatment and the percentage of dead worms at various periods of time after cessation of treatment. Results from 37 rats treated three times daily with an oral dose of 25 mg. per kg. of Hetrazan, and then held seventy-seven days before autopsy, are given in TABLE 9. The results obtained are representative, in general, of the effect of Hetrazan against this infection at what is considered an optional dosage.

TABLE 8

RESULTS OF TREATMENT IN COTTON RATS WITH HETRAZAN TWICE DAILY FOR 4 WEEKS; AUTOPSIES ON THE 4TH, 6TH AND 8TH WEEK

Rat No.	Dose mg. kg.	Microfilariae per 100 fields, weeks										Week of autopsy	Dead worms at autopsy					
		0	1	2	3	4	5	6	7	8	No worms found		100 %	50 to 95%	Less than 50%	None dead		
623	50 O	52	0	0	0	0					4th	X						
601	25 I	34	1	0	1	0					4th				X			
600	25 I	64	0	3	1	0					4th				X			
606	50 O	52	1	0	0	0					4th				X			
607	50 O	108	2	0	0	0					4th				X			
615	25 I	22	1	2	1	4					4th						X	
619	50 O	288	1	1	0	0					4th					X		
593	25 I	22	0	0	0	0	2	1			6th		X					
624	25 I	88	0	2	1	0	3	4			6th							
592	25 I	52	0	1	0	0	0	14			6th						X	
620	50 O	104	0	1	3	0	4	10			6th						X	
621	50 O	608	0	0	0	0	0	0			6th						X	
626	50 O	224	0	0	0	1	4	28			6th						X	
611	50 O	44	0	0	0	0	0	0	0	0	8th	X						
634	50 O	56	0	0	0	0	1	4	8	8	8th			X		X		
616	25 I	152	1	1	1	0	5	4	3	72	8th					X		
599	25 I	64	0	0	0	0	0	4	1	1	8th					X		
618	50 O	68	0	0	0	0	6	6	14		8th					X		
590	50 O	52	1	2	0	0	4	12	28	18	8th					X		
617	25 I	108	0	0	0	1	0	8	1	0	8th					X		
608	50 O	100	0	1	1	0	32	22	72	68	8th							X

I = Intraperitoneal; O = Oral.

It will be noted, in TABLE 9, that in most of the rats the microfilariae were reduced 100 per cent after two weeks of treatment, and that in the remainder the count was nearly negative. In 13 of the rats, the microfilaria count remained negative after cessation of treatment, and at autopsy either all of the worms in these animals were dead or none were found. With one exception, the remaining animals in this series showed relapses in microfilariae of varying degrees after treatment was stopped, and varying numbers of dead worms were found at autopsy. In only three animals were all the adult worms living.

TABLE 9

RESULTS OF TREATMENT IN COTTON RATS WITH 25 MG. PER KG. OF HETRAZAN 3 TIMES DAILY FOR 30 DAYS; AUTOPSIES ON THE 107TH DAY

Rat No.	Microfilariae per 100 fields, days			Microfilariae per cent difference from original count, 107th day	Dead worms at autopsy				
	1	15	107		No worms found	100 %	50 to 95%	Less than 50%	None dead
1406	820	0	0	—100	X				
1511	160	0	0	—100	X				
1540	145	1	0	—100	X				
1553	96	0	0	—100	X				
1403	28	0	0	—100	X				
1421	210	0	0	—100	X				
1414	14	0	0	—100	X				
1402	330	0	0	—100	X				
1409	130	0	0	—100	X				
1418	124	0	0	—100	X				
1425	84	0	0	—100	X				
1325	140	0	0	—100		X			
1368	56	0	0	—100		X			
1512	36	0	0	—100					X
1513	180	0	14	—92.9				X	
1453	36	0	3	—91.7			X		
1408	560	0	68	—87.9			X		
1474	410	0	56	—86.4				X	
1434	370	1	48	—86.1				X	
1410	170	1	24	—85.9			X		
1478	320	0	48	—85.0				X	
1539	200	1	36	—82.0			X		
1530	540	0	112	—79.3				X	
1388	440	0	92	—79.1					X
1536	440	1	96	—78.2				X	
1312	500	1	120	—76.0			X		
1442	280	0	72	—74.3				X	
1508	740	0	390	—47.3					X
1528	900	0	480	—46.3				X	
1424	120	0	72	—40.0				X	
1419	340	0	260	—23.6				X	
1457	400	0	310	—22.5			X		
1498	260	0	220	—15.4				X	
1395	96	1	112	+16.6				X	
1444	320	0	420	+37.5				X	
1480	200	2	280	+40.0				X	
1525	280	0	400	+42.9				X	

The above results demonstrate, as was indicated from earlier experiments, that Hetrazan produces a slow lethal effect against adult worms in cotton rats and does not affect all of them to an equal degree simultaneously. Holding some of the rats listed in TABLE 9 for longer periods of time would probably have revealed larger numbers of dead worms, although it is not suggested that 100 per cent of the worms in all animals would eventually have died from the effects of the drug. The factors which control the percentage kill in individual animals undergoing like dosage with Hetrazan are not known. Various stages of death and disintegration of dead worm tissue have been noted at different periods of time following cessation of treatment, ranging from motionless but otherwise normal-appearing worms to nearly completely decayed worm tissue enveloped in exudate. The complete absence of worms in some animals after long periods have elapsed from cessation of treatment to autopsy suggests that absorption of dead worm tissue eventually occurs. In some rats where no worms were found after treatment, fragments of what appeared to be cuticle were found singly or in small clumps in the pleural cavity. Their positive identification was, however, not possible.

It has been mentioned previously that microfilariae may recur in the peripheral blood following cessation of treatment with Hetrazan or other piperazine compounds. As demonstrated in TABLE 9, the degree of recurrence does not bear a direct relationship to the number of dead worms found at autopsy in every case. Rat 1512, for example, maintained a negative microfilaria count for seventy-seven days after treatment and no dead worms were found at autopsy. On the other hand, several of the rats in TABLE 9 which showed marked relapses in microfilariae after treatment, exhibited the majority of adult worms dead at autopsy. This emphasizes again that the first effect of Hetrazan is directly against the microfilariae. When treatment is stopped, the microfilaria count may be negative in the peripheral blood, but the adult worms have not as yet been damaged sufficiently to prevent the liberation of new microfilariae. When the adult worms die eventually, the microfilaria count may once again become quite high in the peripheral blood, and, since no more drug is present in the animal, the embryos may remain for long periods of time even after most or all of the adult worms are dead. The degree of relapse in microfilariae, therefore, is not indicative in every case of how many adult worms have been damaged by the drug, even after long periods of treatment.

**The Effect of Hetrazan on *Dirofilaria immitis* in Dogs.** The reduction of microfilariae in dogs following intraperitoneal or oral treatment with Hetrazan is, in general, not so rapid as occurs in cotton rats. As demonstrated in TABLE 10, however, a very marked reduction usually occurs within one week's time and, thereafter, the count remains low or becomes negative as treatment progresses.

Although not as many dogs have been treated with Hetrazan as have cotton rats, the results obtained are substantially the same. Some animals,



TABLE 10

EFFECT OF ORAL TREATMENT WITH HETRAZAN THREE TIMES DAILY ON  
*Dirofilaria immitis* IN DOGS

Dog No.	Dose mg. kg.	No. days treated	Microfilariae per 0.05 cc., days											Autopsy record	
			1	7	14	21	28	35	42	49	56	63	70		
993	25	10	135	4	1	0	2	0							R
992	25	21	580	65	21	8									R
193	25	23	18	2	0	0	1	1	1	2	1			1	O
194	25	23	7	22	5	2	0	2	9	3	2			0	*
191	25	23	1	0	0	3	0	0	0	1	0			0	O
997	25	30	406	81	9	1	4	0	3						**
996	25	30	61	36	44	24	21	25	23						O
994	25	30	37	0	7	3	2	22	5						O
998	25	30	21	3	9	4	6	8	4						O
995	25	41	45	46	12	3	0	0	3						R
1000	25	44	30	1	0	0	1	0	0	0	0	0	0		R
999	25	53	24	1	2	0	25	5	0	0	13	12			R
262	25	58	235	265	12	14	4	26	16	5	11	134	50		*
260	25	60	734	39	50	12	27	20	18	20	11	2	8		**
222	25	64	735	29	0	0	2	1	1	2	2	1	1		**
221	25	64	378	19	1	2	1	0	4	2	1	0			*
259	35	27	232	17	14	7	3	3							*
261†	50	27	209	58	124	88	75	43							**

R = Returned to owner, not autopsied.

O = No worms found.

\* = All worms alive.

\*\* = Some or all worms dead.

† = Twice daily.

particularly those with high initial microfilaria counts (997, 260, 222, 261) responded very well to treatment (TABLE 10). The microfilariae were sharply reduced, and upon autopsy dead worms in various stages of disintegration were found in the pulmonary artery within the lung. These animals improved in physical condition up to the time of autopsy and, to all intents, were cured of their infections.

Other dogs, however, as illustrated in TABLE 10, showed marked reductions in microfilariae but no dead worms at autopsy.

The absence of adult worms at autopsy in dogs following treatment has not been considered conclusive proof that an effect against microfilariae was obtained. In contrast to cotton rats, some untreated dogs which show circulating microfilariae reveal no adult worms at autopsy in the heart or lungs. No explanation is offered for this situation, but it does occur and must be taken into consideration when evaluating filaricidal activity in dogs.

Unfortunately, five of the dogs included in TABLE 10 could not be brought to autopsy, since their owners requested their return after treatment. The success of treatment in four of these dogs, as measured by the reduction in microfilariae, was good, but no conclusions can be drawn relative to the fate of the adult worms.

The data obtained from dogs, thus far, indicate that Hetrazan may prove useful in the treatment of heartworm infestations, particularly in dogs with initial high microfilariae counts. It is well tolerated in therapeutic doses (25 mg. per kg. orally).

**Comparison of Hetrazan With Other Filaricides in Cotton Rats and Dogs.** The piperazines, and Hetrazan in particular, are the only filaricides which have been shown to produce an effect in cotton rats and dogs when administered orally in well tolerated doses. Hetrazan differs from antimonials,<sup>1, 2, 3</sup> arsenicals,<sup>5</sup> and the cyanine dyes<sup>4</sup> in its effects, in that the microfilariae are attacked immediately and the adult worms are killed slowly. The antimony derivatives thus far shown to be effective in cotton rats,<sup>1, 2, 3</sup> have been administered in high parenteral doses and kill the adult worms without affecting the microfilariae *per se*. Similarly, the cyanines<sup>4</sup> and *p*-[bis-(carboxymethylcapto)-arseno]-benzamide affect the adult worms quickly without reducing the microfilaria count.

In filaria-infected dogs, Fuadin is claimed to kill a large proportion of the adult worms,<sup>7</sup> and Otto and Maren<sup>5</sup> have demonstrated that *p*-[bis-(carboxymethylcapto)-arsino]-benzamide will also kill adult *Dirofilaria*.

Although Hetrazan does not kill adult worms as quickly as any of the above mentioned compounds, the end results obtained in many animals are essentially the same, in that the microfilariae are eventually killed. Its additional advantages are that it is effective orally, is highly microfilaricidal, and is well tolerated in therapeutic doses. In cotton rats and dogs, its disadvantages are that it must be administered frequently and for relatively long periods of time, and its effects against adult worms in these animals are not 100 per cent predictable.

## SUMMARY

Data are presented to illustrate the filaricidal effects of various piperazine compounds, particularly 1-diethylcarbamyl-4-methylpiperazine hydrochloride (Hetrazan) in cotton rats and dogs. In cotton rats, a number of piperazines produce immediate and sharp reductions in circulating microfilariae when administered intraperitoneally or orally. Relapses may or may not occur after cessation of treatment.

Hetrazan is also effective against adult filariae in cotton rats and dogs. The optimal dosage appears to be 25 mg. per kg. administered orally, three times daily, for thirty days. In some animals, all of the adult worms are eventually killed, while in others only a partial kill is obtained.

A comparison of the effectiveness of Hetrazan with various other filaricidal compounds is given.

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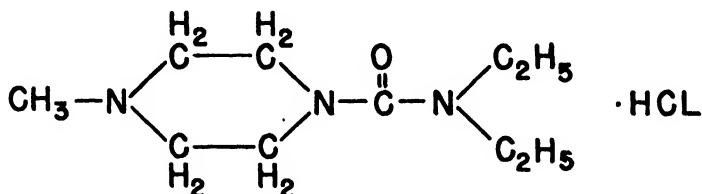
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# SOME TOXICOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF 1-DIETHYLCARBAMYL-4-METHYLPIPERAZINE HYDROCHLORIDE, HETRAZAN

By B. K. HARNED, RAYMOND W. CUNNINGHAM, SYBELLA HALLIDAY,  
R. E. VESSEY, N. N. YUDA, MARY C. CLARK, AND Y. SUBBAROW

*Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.*

Some of the pharmacological properties of a new filaricide shown by Hewitt and coworkers<sup>1, 2</sup> to be highly active against naturally acquired infections have been studied in seven species of laboratory animals. The compound, 1-diethylcarbamyl-4-methylpiperazine hydrochloride, also referred to as Hetrazan and 84L, has a molecular weight of 234.6<sup>3</sup> and possesses the following structural formula:



It is a colorless crystalline solid highly soluble in water. The pH of a 1.0 per cent solution is 4.1. In these experiments, however, the solutions were always adjusted to pH 7.4.

## RESULTS

### Acute Toxicity

**SINGLE DOSES.** *Mice and Rats.* The dose-effect relations have been determined in albino mice and rats after oral, intraperitoneal, and intravenous administrations. TABLE 1 summarizes the data from 238 mice and 260 rats. Values for the L.D.<sub>50</sub> in mg. per kg. were: orally, mice 660, rats 1380; intraperitoneally, mice 248, rats 465; intravenously, mice 82, rats 150. The 19/20 fiducial limits are narrow and the slopes of the mortality curves are steep. Toxic doses produced convulsions which were predominantly tonic. The convulsant dose, however, was considerably below the fatal dose.

*Guinea Pigs, Rabbits, and Cats.* The upper limits of tolerance were determined only in mice and rats; nevertheless, in other species, rather large doses failed to produce serious reactions. Ten guinea pigs given 50 mg. per kg. intraperitoneally showed no symptoms. No objectionable reactions were observed in 15 rabbits given 100 mg. per kg. by the same

TABLE 1

THE ACUTE TOXICITY OF 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HCl, HETRAZAN (84L)

Oral dose mg./kg.		300	400	500	600	700	800	900	1000	1200	1500	2000	2500	mg./kg.	L.D. <sub>50</sub> 19/20 Fiducial limits
Mice	No. injected No. dead			10 0	16 7	16 12	10 8		4 4					660	611-713
Rats	No. injected No. dead	9 0			7 1			10 2	6 0	16 4	15 10	32 17	4 4	1380	1182-1620
Intraperitoneal dose mg./kg.		100	200	225	250	300	400	600							
Mice	No. injected No. dead	10 0	46 1	20 10	24 19	6 5									
Rats	No. injected No. dead		8 0			68 17	20 7	20 15							
Intravenous dose mg./kg.		50	75	100	125	150	175	200							
Mice	No. injected No. dead	14 0	24 9	30 21	6 6										
Rats	No. injected No. dead			4 0	10 1	20 11	10 6	5 5							
															82
															150
															75-90
															139-162

Weight range in grams: mice, 18-25; rats, 100-250.

route. Twenty-five mg. per kg. given intraperitoneally to 6 cats and 50 mg. per kg. given to 3 cats caused vomiting in six to ten minutes. The only other symptom noted was slight drowsiness.

*Dogs.* Serious reactions were not observed in unanesthetized dogs which had received 100 mg. per kg. orally or intraperitoneally, or 25 mg. per kg. by rapid intravenous injection. Some of the symptoms produced by graded doses of 84L in 38 unanesthetized dogs have been recorded in

TABLE 2  
TOXICITY OF 84L IN UNANESTHETIZED DOGS

No. of dogs	Dose mg./kg.	Route of administration	Symptoms†			
			Nausea	Emesis	Respiratory stimulation	Shivering
11	5	intravenous†	1	1	11	0
2	10	intravenous†			2	0
4	20	intravenous†	3	2	4	0
1	25	intravenous†	1		1	1
1	50	intravenous†	1	1	1	1***
2*	100	intraperitoneal	2	2		2
5	50	oral**	4	3		1
12	100	oral**	8	8		1
1	200	oral**	1			1

† The intravenous injections were completed within one minute.

‡ Numbers indicate the dogs affected.

\* These dogs were given 100 mg./kg. twice daily for two days.

\*\* Dogs were fed two to five hours before dosing.

\*\*\* Severe muscular tremors.

Respiratory stimulation lasted about one minute.

Shivering lasted from thirty to sixty minutes.

Vomiting was never accompanied by continued retching and malaise.

Recovery was excellent in all dogs.

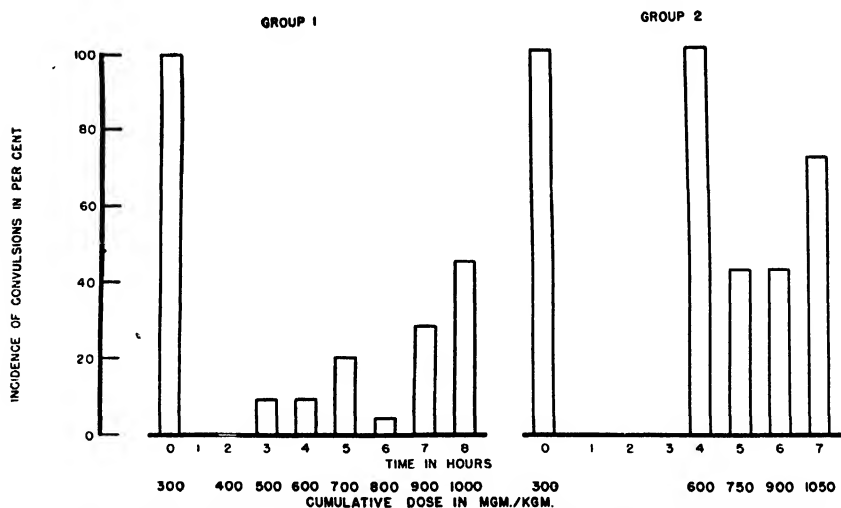
TABLE 2. If reactions occurred, they were, in order of frequency, nausea, emesis, and muscular tremors. These tremors closely approximated those of a dog shivering from a low temperature. In addition to the above reactions, the intravenous injections produced a stimulation of the respiration which lasted from one to three minutes. Oral doses of 50 mg. per kg. and above frequently produced emesis, but the presence of food in the stomach decreased the incidence. Oral doses of 25 mg. per kg. administered with food were always retained.<sup>1</sup>

**MULTIPLE DOSES.** Since Hewitt's antifilarial data<sup>1, 2</sup> indicated the necessity for frequent administration of 84L, it was desirable to study the rate of its destruction or excretion. For this study, experiments were designed to give rats and mice repeated injections at a rate which barely exceeded the capacity of the animal to eliminate the compound. The criteria were the incidence of convulsions and the percentage of mortality.

In rats, the intraperitoneal L.D.<sub>50</sub> was 465 mg. per kg.; therefore, in the tolerance studies, a single intraperitoneal dose of 300 mg. per kg. was given at zero hour with additional 100 mg. per kg. doses at hours 2, 3, 4,

5, 6, 7, and 8. The total dose for the period was 1000 mg. per kg. which, if given at one time, should have produced a mortality of 98 per cent. The observed mortality of the initial dose of 300 mg. per kg. was 31 per cent, while the succeeding doses increased this value only 5 per cent.

The incidence of convulsions provided a delicate indicator of the rate of elimination of the compound (FIGURE 1). At 300 mg. per kg., 100 per cent of the animals convulsed, but after an interval of two hours an additional dose of 100 mg. per kg. produced no convulsions. The incidence after the third and fourth doses was 9 per cent in each case, and, after the eighth dose, 45 per cent. Although the total dose given to these rats amounted to 1000 mg. per kg., no increment subsequent to the initial dose produced convulsions in all of the rats, which would have been the case had the concentration of 84L risen to 300 mg. per kg. Thus, it appears that under the above conditions the rat is capable of eliminating approximately 100 mg. per kg. per hour.



THE CONVULSANT EFFECT IN RATS OF MULTIPLE INTRAPERITONEAL DOSES OF 84 L

FIGURE 1. The incidence of convulsions during cumulative dosing provides a criterion for the rate of elimination of the compound. There were thirty-two rats in group I and thirteen in group II, ranging in weight from 200 to 250 grams.

Obviously, the validity of this calculation rests on the assumption that the rat does not become resistant to the convulsant action of the compound. This question has been answered by the data on Group II in TABLE 3. In this series, the original dose of 300 mg. per kg. was repeated after 4 hours. The incidence of convulsions was again 100 per cent and the mortality 22 per cent. When additional doses of 150 mg. per kg. per hour were given on the fifth, sixth, and seventh hours, the incidence of the convulsions rose from 43 per cent on the fifth hour to 71 per cent on the seventh.

These data show that rats do not become resistant to the convulsant action of 84L.

A similar experiment on mice has yielded data on the same order. Thus, the administration of four times the L.D.<sub>50</sub> over a period of 8 hours produced in mice a total mortality of 27 per cent (FIGURE 2). The rate of elimination in mg. per kg. per hour appeared to be greater in mice than in rats.

TABLE 3

THE EFFECTS OF MULTIPLE INTRAPERITONEAL DOSES OF 84L REPEATED AT SHORT INTERVALS TO RATS

Interval subsequent to initial dose	No. of rats injected	Dose, mg./kg.		Incidence of convulsions per cent	Mortality per cent
		Single	Cumulative		
hours					
0	45*	300	initial dose	100.	31.1

*Group I\*\**

2	22	100	400	0.	0.
3	22	100	500	9.1	4.7
4	21	100	600	9.5	0.
5	21	100	700	19.0	0.
6	21	100	800	4.7	0.
7	21	100	900	28.6	0.
8	11	100	1000	45.5	0.†

*Group II*

4	9	300	600	100.	22.2
5	7	150	750	43.	0.
6	7	150	900	43.	0.
7	7	150	1050	71.4	42.8†

Range of weight in grams: 200 to 250.

\* The survivors were divided into two groups, I and II.

\*\* Combined results of a group of 10 and a group of 12 dosed on different days.

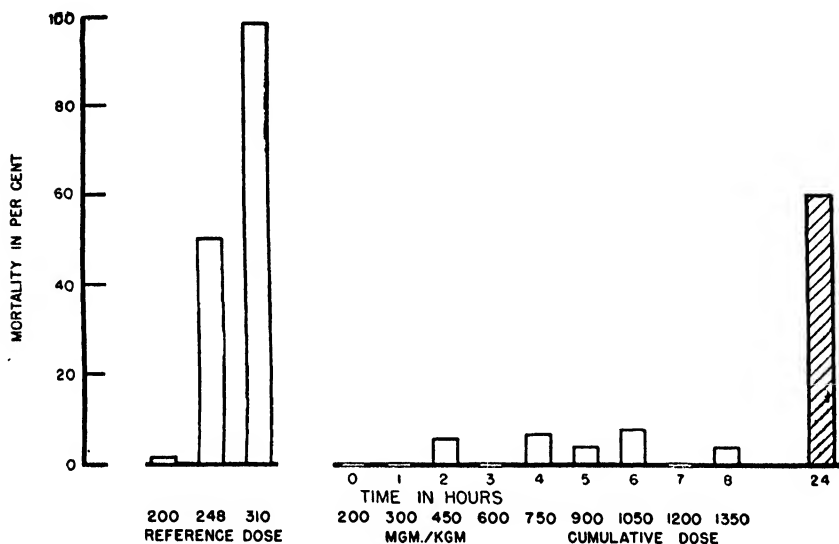
† Twenty-four hours later, all survivors were in good condition.

Etherized dogs tolerated 60 to 70 mg. per kg. of 84L given intravenously during a period of one hour. Dog No. 548 readily tolerated ten 5 mg. per kg. doses during a period of 90 minutes, and dogs Nos. 546 and 547 each received seventeen 5 mg. per kg. doses in 80 minutes without endangering the respiration. Dog No. 549 (TABLE 7) tolerated seven 10 mg. per kg. doses during a period of 60 minutes but developed respiratory failure when the eighth dose was given on the seventieth minute. This dog was maintained without difficulty on artificial respiration. Dog No. 540 received four 20 mg. per kg. doses in 40 minutes. The fifth dose which brought the total to 100 mg. per kg. in 52 minutes



produced respiratory failure. Artificial respiration maintained this animal in a satisfactory condition and 20 minutes after the fifth dose another injection of 20 mg. per kg. was made without producing circulatory failure.

Unanesthetized dogs have been given 100 mg. per kg. intraperitoneally twice daily for 2 days without producing symptoms more severe than vomiting and mild muscular tremors (TABLE 2). One hour after the injections, the animals appeared normal.



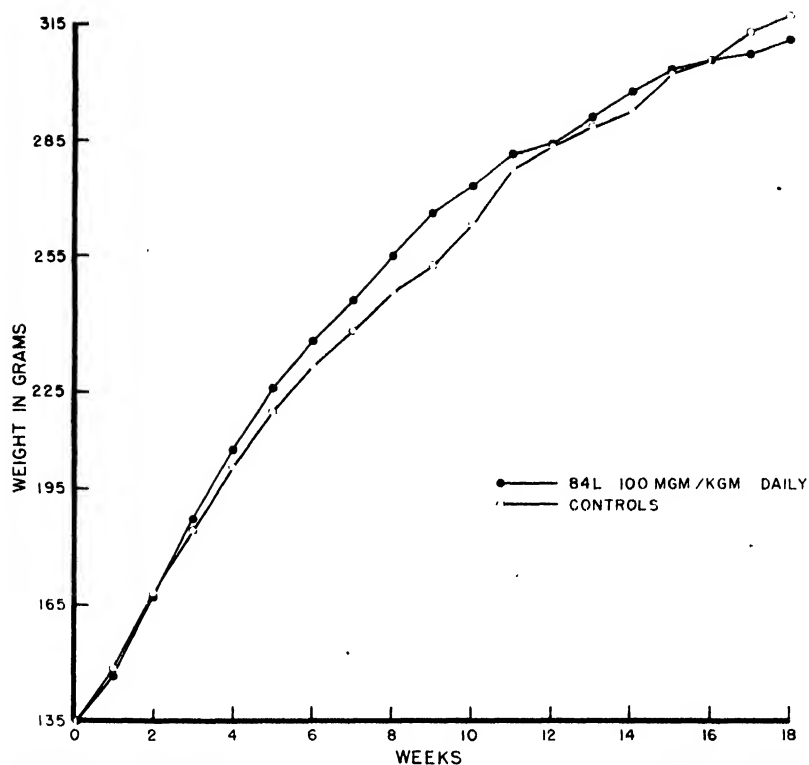
THE EFFECT IN MICE OF MULTIPLE INTRAPERITONEAL DOSES OF 84 L

FIGURE 2. Reference doses represent single injections. Death from these single injections usually occurred within thirty minutes. There were thirty mice in the multiple-dose series.

### Chronic Toxicity

**Rats.** The intraperitoneal injection of 100 mg. per kg. of 84L, 5 days per week for 18 weeks, did not affect the rate of growth or produce any unfavorable reactions in male rats (FIGURE 3). The control and dosed groups each started with 20 animals. After 18 weeks, there were 15 in the control and 14 in the dosed group. At the end of the series of doses, the mean hematological findings on 10 rats from each group were: (1) hemoglobin in grams per 100 cc., controls 13.1, dosed 13.5; (2) red blood cells per cu.mm., controls 8.3 million, dosed 8.7 million; (3) white blood cells per cu.mm., controls 14.4 thousand, dosed 19.3 thousand; (4) lymphocytes, controls 61 per cent, dosed 79 per cent; (5) neutrophils, controls 34 per cent, dosed 19 per cent; (6) eosinophiles, controls 3.7 per cent, dosed 1.0 per cent. The pathologist, Dr. F. I. Dessau, reported that there were no differences between the groups.

**Rabbits.** A group of 15 rabbits was given, intraperitoneally, 50 mg. per kg. for 15 weeks. The growth of the group was not significantly



## GROWTH CURVES

INTRAPERITONEALLY, 5 DAYS PER WEEK

## MALE RATS

FIGURE 3.

Number of rats		Start	End
Control	.....	20	16
Dosed	.....	20	14

modified (FIGURE 4). During the period, 4 animals died in the control group and 7 in the dosed, but the distribution of deaths does not suggest that they were due to chronic effects of the drug. After 66 doses during 99 days, the hematological data revealed no difference between the control and dosed groups. The average results for the groups were: (1) hemoglobin in grams per 100 cc., controls 11.1, dosed 11.0; (2) red blood cells per cu.mm., controls 6.1 million, dosed 5.8 million; (3) white blood cells per cu.mm., controls 8.6 thousand, dosed 10.3 thousand; (4) lymphocytes, controls 69 per cent, dosed 70 per cent; (5) neutrophils, controls 28 per cent, dosed 26 per cent.

**Dogs.** Hewitt<sup>1</sup> has never observed any evidence of chronic toxicity in a large series of dogs. Five of his dogs were given, intraperitoneally, 50 mg. per kg. twice a day for 13 days, and 2 animals in his series were dosed orally with 25 mg. per kg. three times a day for 64 days. At the end of the period of dosing they were sacrificed and examined by the pathologist, Dr. F. I. Dessau. Again, his findings were negative.

*Chicks.* Robbins's observation<sup>4</sup> that 2,4-dinitrophenol produced cataracts in the eyes of chicks prompted us to subject 84L to a similar test. A group of eighteen 3-day-old White Rock chicks were divided equally into three balanced groups. Group one received a diet which contained 0.25 per cent 84L, group two, 0.25 per cent 2,4-dinitrophenol, and group three, normal diet. Chicks on 2,4-dinitrophenol developed cataracts during the first 24 hours. The chicks in group one were continued on the 84L diet for 15 days, and there was never any evidence of changes in the lens. At the end of the experiment the lenses were sectioned and the pathologist, Dr. E. Woll, reported that they were normal. The chicks fed 84L grew at the same rate as the controls in group three, and in appearance the individuals of the two groups were indistinguishable.

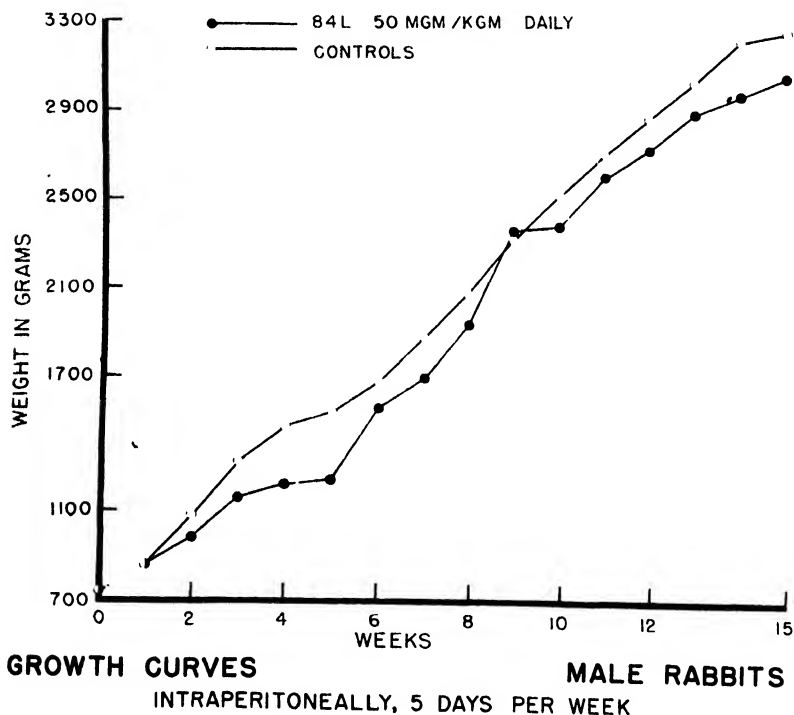


FIGURE 4.

Number of rabbits	Start	End
Control .....	15	11
Dosed .....	15	8

### Miscellaneous Observations

*General Behavior.* Intraperitoneal doses of 50 mg. per kg. to dogs, cats, rats, and rabbits produced few symptoms. The dogs and rats were more sensitive to loud noises, but approximately 30 minutes after the

injections many of the animals in all of the species tested appeared to be more quiet than usual. However, they were not asleep and responded normally to external stimuli.

*Local Anesthesia and Irritation.* Intracutaneous injections of 0.1 cc. doses of 1.0 per cent 84L in guinea pigs gave no evidence of local anesthesia or irritation. Likewise, repeated application of this concentration of the compound to the eyes of cats gave negative results.

*Isolated Intestine.* The activity on isolated rabbit ileum is of a low order. Concentrations of 1:100,000 in Tyrode's solution were required to give a perceptible relaxation of normal or spastic strips. It produced no effect on the guinea pig intestine.

*Isolated Uterus.* In concentrations of 1:100,000, 84L produced no effect on the isolated uterus from the rabbit or the rat. Virgin guinea pig uteri responded with weak contractions to a 1:100,000 concentration. In lower concentrations, the response was barely perceptible or absent.

*Antihistaminic Action.* The antihistaminic action on the guinea pig ileum was barely detectable, and amounted to 1/2000 to 1/10,000 of the activity of some of the clinically used compounds. Ten guinea pigs were injected intraperitoneally with 50 mg. per kg. of 84L and 30 minutes later were subjected to a standardized spray of histamine. Nine animals convulsed in 3 minutes and of these 1 died. One guinea pig withstood the spray for 10 minutes with no symptoms other than dyspnea. A retest of this guinea pig one and one-half hours after 84L produced convulsions

TABLE 4  
EFFECT OF 84L ON THE BLOOD SUGAR OF ADULT MALE RATS

Rat No.	Weight grams	Intraperitoneal dose of 84L mg./kg.	Hours after 84L		
			Control	1	2
				'True' sugar in terms of mg. of glucose per 100 cc. of blood	
1	204	250*	88	104	88
2	202	250*	89	116	91
3	192	50	71	77	72
4	202	50	76	77	67
5	206	25	80	79	74
6	220	25	89	74	77
7	212	0	89	76	75
8	202	0	84	77	79
9	186	0	77	75	73
10	182	0	82	68	67

Rats were fasted sixteen to seventeen hours.

\* Muscular tremors were observed.

in 7 minutes. A group of ten control guinea pigs all convulsed in 3 minutes and 3 died. From these data, we conclude that 84L does not exaggerate the action of histamine but exerts little protection against it.

*Eye.* Six cats were given, intraperitoneally, 25 mg. per kg. and three, 50 mg. per kg. of 84L. No evidence of myosis or mydriasis was observed. The local application of a 1.0 per cent solution produced no change in the pupil.

*Blood Sugar.* In subconvulsant doses, 84L had no effect upon the blood sugar (TABLE 4).

*Diuretic Action.* By the Lipschitz rat assay,<sup>5</sup> the diuretic potency is 17.5 times that of urica or about one-half the effectiveness of caffeine.

*Analgesia.* In rats, the compound produced evidence of a mild analgesia. The action was less than that produced by aminopyrine.

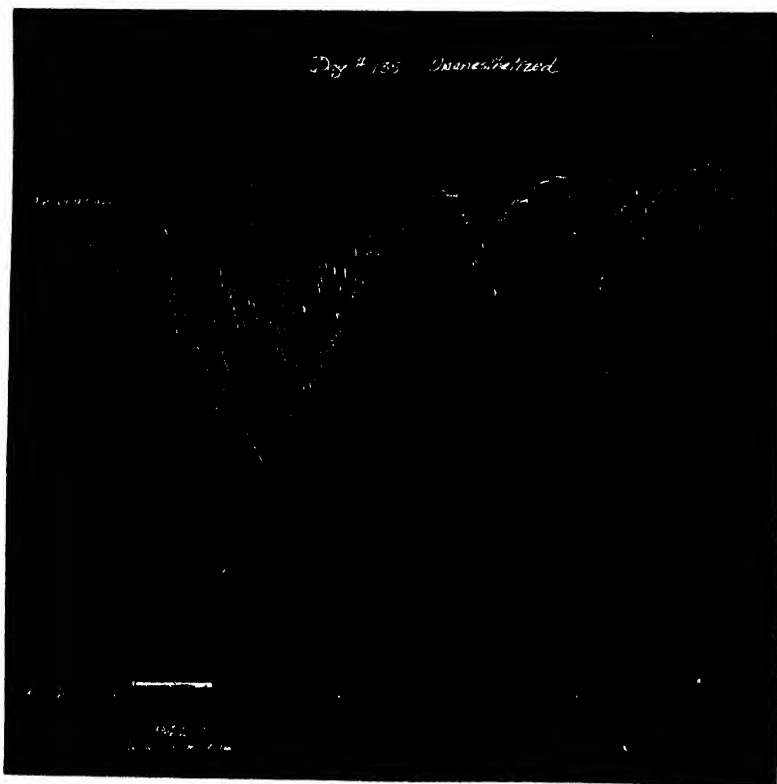


FIGURE 5. Typical record of respiratory movements in an unanesthetized dog after an intravenous injection of 84L. The signal line marks the duration of the injection. The respiratory movements were relayed to the kymograph by a thread attached halfway between the umbilicus and the end of the sternum.

*Respiration.* Changes in respiration have not been observed except after intravenous doses. In unanesthetized dogs, the intravenous injection of 5 mg. per kg. in 8 seconds stimulated the rate and depth of respiration for about a minute. Within 2 to 3 minutes from the start of the injection, the respiration was normal (FIGURE 5). When the same dose was injected over a period of 60 seconds, the stimulation was of a lower order and of shorter duration. The injection of 20 mg. per kg. in 18 seconds produced an intense stimulation of respiration which lasted about a minute. In 3 minutes, the animal appeared to be normal. In etherized dogs, the respiratory changes were much less prominent.

Intravenous doses of 2 to 15 mg. per kg. inhibited the respiration of normal and anesthetized rabbits. In unanesthetized rabbits, the rapid injection of 5 mg. per kg. produced an apnea of 5 to 10 seconds' duration. Recovery was complete in 3 minutes. A dose of 2 mg. per kg. injected in 60 seconds decreased the amplitude and slowed the respiration for 30 seconds. Twenty-five mg. per kg. injected in 1 minute killed one of two rabbits.

In anesthetized dogs, lethal doses of 84L produced death by respiratory failure. Many dogs were maintained on artificial respiration long after their normal respiration had ceased.

*Circulation.* Electrocardiograms\* on etherized dogs which had been injected intravenously with 5 to 50 mg. per kg. of 84L revealed minimal changes. The heart rate was increased from one hundred and fifty to one hundred and eighty beats per minute during the hypertensive phase, but returned to the initial rate during the hypotensive phase. Regular sinus rhythm was maintained and such changes in the form of the complex as were noted were those usually associated with rapid heart rates. Electrocardiograms taken during the terminal stages revealed no changes not typical of anoxia.

In etherized animals, the response of the blood pressure to effective doses of 84L resembled those produced by epinephrine. The principal difference noticed was a longer duration of the depressor phase. Doses of 0.1 and 0.5 mg. per kg. were ineffective. Some dogs responded feebly to 1 mg. per kg., but 5 mg. per kg. produced a quick, short-lasting rise in the blood pressure followed by a fall of somewhat longer duration. As the dose was increased, the peak of the rise increased until it reached a maximum at about 20 mg. per kg. The changes in the blood pressure produced by initial doses ranging from 5 to 40 mg. per kg. have been recorded in TABLE 5. The pressor responses were as follows: 5 mg., 32 mm. of Hg; 10 mg., 52 mm. of Hg; 20 mg., 50 and 92 mm. of Hg; 25 mg., 48 mm. of Hg; and 40 mg., 42 mm. of Hg. In the higher doses, the duration of the rise tended to increase, but the most prominent change was the prolongation of the time required for recovery from the depressor phase.

\* The electrocardiographic studies were made by Dr. Maynard B. Chenoweth, Cornell University Medical College.

TABLE 5

THE EFFECT OF INITIAL INTRAVENOUS DOSES OF 84L ON THE BLOOD PRESSURE OF ETHERIZED ANIMALS

Dog No.	Blood pressure control mm. Hg	Dose intra-venous† mg./kg.	Blood pressure			
			Rise		Fall	
			Maximum mm. Hg	Duration minutes	Maximum mm. Hg	Duration minutes
504	136	5	26	0.5	34	1.3
503	165	5	33	0.3	37	1.5
502	152	5	36	0.5	27	2.0
501	120	5	36	0.5	38	2.5
506	133	5	28	0.2	29	2.5
522	142	5	85	0.5	0	—
488	106	10	54	1.0	26	*
A-1	95	10	51	2.5	0	—
A-4	142	10	50	0.5	66	5.0
540	130	20	50	0.5	34	13 plus
A-2	120	20	92	1.2	40	9 plus
545	122	25	46	1.3	48	5 plus
489	112	25	28	1.0	52	*
490	115	25	39	2.0	48	*
491	145	25	91	4.0	0	—
544	156	25	38	1.0	50	5 plus
A-3	104	40	42	0.5	36	35
Cat No.						
498	136	10	12**	1.5	0	—
492	120	25	40	2.0	0	—
Rabbit No.						
495	98	10	42	15.0	0	—
493	68	25	26	3.0	0	—
494	88	25	Fatal Dose			

† The injection was completed in ten to thirty seconds.

\* Fall was interrupted by the injection of another compound.

\*\* This rise was preceded by a fall of 8 mm. of Hg. The duration of the fall was 0.3 minutes.

The leg-plethysmograph readily demonstrated that the sharp rise in the blood pressure produced by intravenous doses of 84L in dogs was accompanied by an equally sharp decrease in the volume of the leg. However, the vasodepressor phase is poorly reflected.

The close similarity between the response of the blood pressure to 84L and to epinephrine, coupled with the observation that this compound exaggerated the action of the latter, suggested that it either inhibited the cardiac vagus or those reactions which destroy epinephrine.

The vasodepressor response to stimulation of the right vagus before and after 84L showed that the intravenous injection of 25 mg. per kg. blocked 25 to 100 per cent of the vagal activity for a period of 30 minutes (TABLE 6). The exaggeration of the pressor response of epinephrine parallels the inhibition of the vagus. Doses of 5 and 10 mg. per kg. of 84L produced essentially no vagal inhibition (TABLE 6). The mechanism of inhibition is interesting since in doses which completely inhibited the cardiac vagus, 84L had no action on the vasodepressor response to acetylcholine.<sup>6</sup>

TABLE 6  
THE INHIBITION OF THE CARDIAC VAGUS BY 84L

Animal No.*	Control vagal stimulat. fall in blood pressure mm. Hg	Dose intravenous mg./kg.	Minutes after injection		
			3	15	30
			Vagal inhibition in per cent		
Cat	498	22	45	18	0
	498	12	0		
	498	16	100	81	81
	492	31	80	61	0
	492	23	56	39	0
Rabbit	493	26	100		19
	493	20	0		
Dog	544	76	100	100	68
	489	64	100	100	46
	545	66	100	48	42
	490	70	85	78	69
	487	56	100	100	100†
	488	60	0		
	488	60	75‡		
	491	100	50	60	25
	542	74	8	0	
	542	73	7		

The right vagus was used. The blood pressure was recorded from the right carotid artery.

\* All animals were anesthetized with ether except dog No. 491. In this animal, the anesthetic was intravenous sodium pentobarbital.

† One hundred per cent inhibition for at least two hours.

‡ Vagal activity was normal at forty-five minutes.

Repeated injections produced a gradual diminution in the responses of the blood pressure and, finally, a complete disappearance of all action (TABLE 7). These data may provide a key to the explanation of the disappearance of the headache which developed in some patients after a few doses.

Dogs with cords sectioned at approximately the second cervical vertebra and all superior connections completely removed showed unmistakably that the vasopressor response of 84L was not dependent upon centers in the brain. After this operation, the vasopressor action of 84L was



TABLE 7

THE EFFECT OF MULTIPLE INTRAVENOUS DOSES OF 84L ON THE BLOOD PRESSURE OF ETHERIZED DOGS

Dog No.	Blood pressure control mm. Hg	Dose		Blood pressure			
				Rise		Fall	
		Intra-venous mg./kg.	Interval minutes	Maximum mm. Hg	Duration minutes	Maximum mm. Hg	Duration minutes
532	142	0.5		0		0	
	142	5.0	7	38	0.5	18	1.5
	147	5.0	5	33	0.5	14	4.0
	146	1.0	5	6	0.5	10	1.5
	145	5.0	5	21	0.5	22	4.5
	144	5.0	6	26	0.5	22	3.5
	145	5.0	5	0		14	6 plus
	142	5.0	6	0		13	5 plus
	139	5.0	30	6	0.5	17	3.0
	150	5.0	60	4	0.5	16	4 plus
548	102	0.5		0		0	
	96	1.0	10	0		0	
	97	5.0	10	35	0.5	33	1.5
	98	5.0	10	30	0.5	38	1.5
	94	5.0	10	25	0.5	28	1.5
	94	5.0	10	28	0.5	29	1.0
	96	5.0	10	23	0.5	16	1.5
	104	5.0	10	9	0.5	12	3.0
	103	5.0	10	4	0.5	15	3 plus
	100	5.0	10	0		20	3 plus
	90	5.0	10	9	0.5	16	3 plus
	90	5.0	10	0		16	16 plus
549	115	0.5		0		0	
	122	1.0	10	2	0.25	0	
	130	10.0	10	36	0.3	46	3.0
	130	10.0	10	30	0.3	45	10 plus
	122	10.0	10	32	0.3	36	2.0
	124	10.0	10	34	0.3	12	1.0
	113	10.0	10	7	0.5	23	5 plus
	93	10.0	10	0		23	3 plus
	90	10.0	10	0		24	3 plus
	86	10.0*	10	0		30	5.0
540	130	20.0		50	0.5	34	13 plus
	127	20.0	13	58	0.5	41	13 plus
	122	20.0	13	52	0.5	37	13 plus
	114	20.0	13	44	0.5	20	13 plus
	102	20.0*	13	10	0.5	12	7
	109	20.0	22	0		0	

\* Respiration failed after this dose. Artificial respiration was continued for the next thirty-two minutes. The cardiovascular system was functioning satisfactorily when the artificial respiration was discontinued.

exaggerated (TABLE 8). Rises of blood pressure of one hundred to one hundred and eighty mm. of Hg were common. Furthermore, the duration of the rise was much longer than in the etherized dogs. After the rise,

there was no fall unless the compound had been given before the blood pressure reached a stable level.

TABLE 8

THE EFFECT OF INTRAVENOUS DOSES OF 84L ON THE BLOOD PRESSURE OF DOGS WITH CORDS SECTIONED AT C-2

Dog No.	Blood pressure Control mm. Hg	Dose		Blood pressure		
				Rise		Fall
		Intravenous mg./kg.	Interval minutes	Maximum mm. Hg	Duration minutes	Maximum† mm. Hg
505	94	25*		24	0.7	38
505	54	10	60	100	8.5	0
505	50	10	60	100	9.0	0
507	71	5		189	4.0	15
507	50	20	20	180	4.5	0
507	50	5	20	164	4.5	0
507	48	20	21	180	7.0	0
507	52	20	22	162	6.0	0
506†	52	20		36	2.0	12
506†	44	5	60	111	3.0	0
506†	46	5	7	90	2.5	0
506†	46	5	5	70	3.0	0
506†	44	5	5	66	3.0	0
506†	46	5	5	54	3.0	0
506†	50	5	5	50	2.0	0
506†	50	5	5	42	2.0	0
522†	77	5		173	5.0	13

† See TABLE 5 for response before section.

‡ When a fall occurred, the recovery was very slow and never complete.

\* After the rise, the blood pressure fell 38 mm. of Hg and remained relatively constant. The blood pressure of this dog probably had not reached its constant level before the injection.

*Cardiovascular Responses in Unanesthetized Dogs.* In five unanesthetized dogs, the rapid intravenous injection (10 seconds) of 5 mg. per kg. of 84L produced an increase in blood pressure which was recorded by direct puncture of the femoral artery. Expressed in mm. of Hg the elevations were: 68, 68, 68, 88, 118. When the same dose was given in 60 seconds, 2 dogs responded with a rise of 31 mm. of Hg. These data indicated that the cardiovascular system of unanesthetized dogs was more sensitive to 84L than that of etherized dogs. Electrocardiograms reflected similar differences. Although the rapid intravenous injection of 5 mg. per kg. of 84L produced insignificant changes in the electrocardiogram of etherized dogs, the same dose in unanesthetized dogs produced a number of changes in the rate and pacemaker location. Sinus arrest for periods as long as 9 seconds, and irregular rapid sinus rhythm interspersed with junctional and ventricular premature contractions were observed. Five minutes after the injection, the electrocardiogram was normal. Doses

of 0.1 to 0.5 mg. per kg. produced no changes or only slight changes in the rate or form of the electrocardiogram.

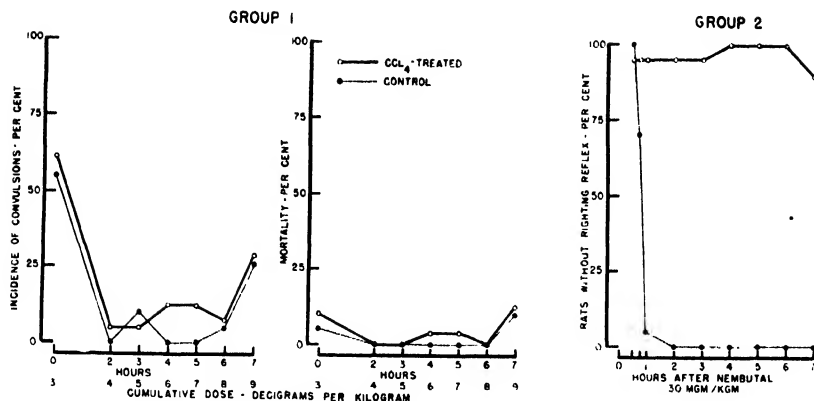
### Fate of Compound

The absence of cumulative effects after multiple doses of 84L (FIGURES 1 and 2) suggested that the animals quickly became highly tolerant or that the compound was rapidly destroyed or excreted. Although a certain tolerance to the vascular action of the drug developed, no comparable action was seen in any other system.

*Liver.* The role of the liver in the destruction was examined by subjecting rats, first, to a series of doses of carbon tetrachloride and then, before hepatic recovery, to multiple doses of 84L. The quantity of 84L used slightly exceeded that which the rat was able to destroy or excrete, so that any limitation in the ability of a key organ to perform its function would be reflected in a greater incidence of reactions in the test-group. Rats weighing from 180 to 210 grams were given orally 0.166 cc. of carbon tetrachloride per 100 grams of body weight. This dose was diluted to 0.5 cc. with corn oil (Mazola) and given on days 1, 2, 3, 4, 5, 7, and 8. The tests for liver damage and 84L catabolism were made on the first day after the last dose of carbon tetrachloride. The function of the liver was tested by following the duration of the anesthesia produced by sodium pentobarbital, a drug which is destroyed principally in the liver.<sup>7</sup> The results shown in FIGURE 6 demonstrate that a dose of 30 mg. per kg. of sodium pentobarbital anesthetized 95 to 100 per cent of the control and carbon tetrachloride-treated rats. Duration of the anesthesia as measured by the righting reflex was 1 hour in the control group and 7 hours in the treated group. The administration of 84L in multiple graduated doses to rats treated similarly with carbon tetrachloride gave little evidence of an exaggeration of the effects seen in normal rats (FIGURE 6). These data suggest that the liver is unimportant in the detoxification of 84L.

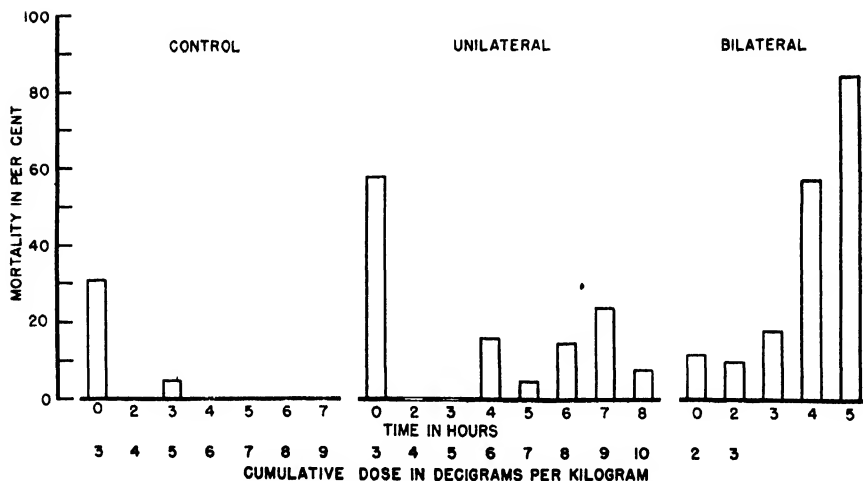
*Kidneys.* The administration of multiple doses of 84L to unilaterally and bilaterally nephrectomized rats demonstrated the importance of the kidney in the elimination of this compound (FIGURE 7, table 9). In a group of 60 rats, the left kidney was removed and after 4 or 5 days the animals were injected intraperitoneally with repeated doses of 84L. The schedule of doses was identical with that of group I in TABLE 3. A comparison of these data (TABLE 9) with those from normal rats (TABLE 3) is typically illustrated by the mortality figures. Mortality in the rats with one kidney was 78 per cent, while in the unoperated group it was 34 per cent.

There were two groups of bilaterally nephrectomized rats (TABLE 9). In one group, the total nephrectomy followed 6 days after the removal of the left kidney. The 84L was administered 24 hours after the last operation. The initial dose was 200 mg. per kg. with subsequent doses



### THE EFFECT OF LIVER DAMAGE ON THE TOXICITY OF CUMULATIVE DOSES OF 84L IN RATS

FIGURE 6. A comparison of the effect of multiple intraperitoneal doses of 84L on the incidence of convulsions and mortality in normal rats and in rats with livers damaged by carbon tetrachloride administered in Mazola oil, 1 cc. diluted to 3 cc. The dose of this mixture was 0.5 cc. per 100 grams of body weight given orally on days 1, 2, 3, 4, 5, 7, and 8. Tests were made on the ninth day. The duration of pentobarbital anesthesia was taken as a criterion of liver function. Each of the two groups contained twenty rats ranging in weight from 140 to 170 grams.



### THE EFFECT IN NEPHRECTOMIZED RATS OF MULTIPLE INTRAPERITONEAL DOSES OF 84L

FIGURE 7. The effect of multiple doses of 84L on the mortality in unilaterally and bilaterally nephrectomized rats. The number of rats in each group was: control, thirty-two (200-250 grams); unilaterally nephrectomized, twenty-eight (170-200 grams); and bilaterally nephrectomized, sixty (170-200 grams).

TABLE 9  
THE MORTALITY PRODUCED BY 84L GIVEN IN MULTIPLE DOSES TO  
NEPHRECTOMIZED RATS†

Group*	Interval subsequent to initial dose, hours	No. of rats in- jected	Dose		Mortality	
			Single mg./kg.	Cumu- lative mg./kg.	Per dose per cent	Cumu- lative per cent
I	0	60	300	initial dose	58	58
Unilateral neph- rectomy 4-5 days before initial dose	1	—	—	—		
	2	25	100	400	0	58
	3	25	100	500	0	58
	4	25	100	600	16	65
	5	21	100	700	5	67
	6	20	100	800	15	72
	7	17	100	900	24	78
	8	13	100	1000	8	80
II**	0	28	200	initial dose	18	18
Left kidney re- moved 6 days before initial dose. Right kid- ney removed 24 hours before initial dose	1	—	—	—		
	2	23	100	300	17	33
	3	19	100	400	26	50
	4	14	100	500	71	86
	5	4	100	600	100	100
III	0	15	200	initial dose	0	0
Total nephrectomy ½-2 hours be- fore initial dose	1	—	—	—		
	2	15	100	300	0	0
	3	15	100	400	7	7
	4	14	100	500	44	47
	5	8	100	600	75	87

† Effect of 84L in normal rats is summarized in TABLE 3, group I.

\* Weight range in grams: Groups I and II, 170-200; Group III, 204-240.

\*\* The mortality in control animals for group II was 15 per cent, in group III, zero per cent.

of 100 mg. per kg. given on the second, third, fourth, and fifth hours. After an accumulated dose of 600 mg. per kg. on the fifth hour, the mortality was 100 per cent. In normal rats (TABLE 1) a single dose of 600 mg. per kg. produced a mortality of 75 per cent. In the completely nephrectomized rats (TABLE 9) an accumulated dose of 400 mg. per kg. killed 14 of the 28 which compared favorably with an L.D.<sub>50</sub> of 465 mg. per kg. in normal rats. The survival of 13 totally nephrectomized rats given no compound was 85 per cent on the first day after the operation, 54 per cent on the second day, and 8 per cent on the third day.

*Excretion.* Although the data indicate that the elimination of the compound is accomplished principally by the kidney, the form in which it is excreted has not been determined. No practical chemical test for small quantities of the compound has been devised. However, a bioassay

on filaria-infected rats indicated that the equivalent of 63 per cent of a 300 mg. per kg. intraperitoneal dose was excreted during the first 23 hours. In spite of the fact that nothing is known about the metabolic fate of 84L, the low antifilarial activity of its relatives<sup>3</sup> strongly suggests that, in the rat, most of the compound is excreted unchanged.

### SUMMARY AND CONCLUSIONS

1. 1-Diethylcarbamyl-4-methylpiperazine hydrochloride, also known as Hetrazan and 84L, has a low toxicity and few side reactions.

2. The intraperitoneal L.D.<sub>50</sub> in mice was 248 mg. per kg. and in rats 465 mg. per kg. The oral L.D.<sub>50</sub> in mice was 660 mg. per kg. and in rats 1380 mg. per kg.

3. Mice, rats, rabbits, and dogs readily tolerated intraperitoneal injections of 100 mg. per kg.

4. Daily intraperitoneal doses of 50 mg. per kg. in rabbits and 100 mg. per kg. in rats, 5 days per week for 15 weeks produced no evidence of toxicity. Twenty-five mg. per kg. three times a day orally for 2 months produced no evidence of toxicity in dogs.

5. The compound was not irritating, produced no local anesthesia, no effect upon the eye, no effect on the isolated uterus or intestine, and no effect on the blood sugar. It was mildly diuretic and analgesic. Intravenous doses of 2 to 25 mg. per kg. in unanesthetized dogs stimulated the respiration. The heart and blood pressure were not affected by rapid intravenous injections of 0.5 mg. per kg., but larger intravenous doses in unanesthetized dogs produced some deviation from normal.

6. The compound was rapidly excreted by the kidney. In rats and mice, the rate per hour was approximately one-third of the intraperitoneal L.D.<sub>50</sub>.

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# THE TREATMENT OF FILARIASIS BANCROFTI WITH 1-DIETHYLCARBAMYL-4-METHYLPIPERAZINE HYDROCHLORIDE (HETRAZAN)\*

By D. SANTIAGO-STEVENSON, J. OLIVER-GONZALEZ, AND  
REDGINAL I. HEWITT

*Departments of Clinical Medicine and Medical Zoology, School of Tropical  
Medicine, San Juan, Puerto Rico, and Lederle Laboratories Division,  
American Cyanamid Company, Pearl River, N. Y.*

The attempts to eradicate infections with *Wuchereria bancrofti* by means of chemotherapeutic agents have been greatly intensified during the last five years. The National Research Council listed over one hundred and twenty compounds which by 1945 had been given clinical trial in human filariasis.<sup>1</sup> Of all drugs used, only the antimony compounds appear to have beneficial effects. Of these, the pentavalent antimonial, neostibosan, has had the best antagonistic action on the filaria worms.<sup>2, 3</sup> Antimony compounds apparently act on the adult filariae, the circulating embryos disappearing gradually from the blood stream. The complete disappearance of the embryos takes place from a few months, in some patients, to longer periods of time in others. A drug had to be found which possessed a more marked and rapid effect on the microfilariae and the adult worms.

Hewitt and his co-workers<sup>4</sup> have recently reported on the marked filaricidal action of 1-diethylcarbamy-4-methylpiperazine hydrochloride on naturally acquired filarial infections in cotton rats and dogs. The drug was given orally and the therapeutic doses utilized on these animals failed to produce evidence of toxic effect on the host. Complete studies on the toxicity of this drug in animals were performed,<sup>5</sup> followed by toxicity studies on normal healthy adults<sup>6</sup> to whom were administered doses as high as 8 mg. per kg. of body weight, without any serious or lasting untoward reactions. Clinical trial of the drug in human filariasis was indicated next. Some of the possibilities to be determined were toxicity to man and, if filaricidal in the human host, the optimal dosage and the duration of treatment.

\* Part of the material in this article appeared in English in the Journal of the American Medical Association. The drug for these studies was supplied by the Lederle Laboratories and Calco Chemical Divisions, American Cyanamid Company. In the medical care of the patients, the authors were assisted by Drs. E. J. Marchand and C. J. Margarida. The chemical studies were performed by Dr. R. Ruiz Nazario.

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<sup>6</sup> Hewitt, R., Personal communication.



TABLE 1  
EFFECT OF 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HYDROCHLORIDE ON CIRCULATING MICROFILARIAE OF  
*Wuchereria bancrofti*.

Patient No., hospital No.	Age	Sex	Amount of drug administered orally		Number of microfilariae in 60 cu.mm. of blood							
			Mgs. per kilo t.i.d.	Length of R <sub>x</sub>	Before R <sub>x</sub>	2nd day of R <sub>x</sub>	Follow-up examinations					
							Days after R <sub>x</sub>	No. of micro- filariae	Days after R <sub>x</sub>	No. of micro- filariae	Days after R <sub>x</sub>	No. of micro- filariae
1 A 10,300	30	M	2	17	30	0	83	0	122	0	150	0
2 A 10,062	30	M	2	18	7	0	30	0			160	0
3 A 8,862	26	M	2	16	220	1	83	1			150	2
4 A 10,317	27	M	2	22	1	0	83	0	122	0	150	0
5 A 7,465	25	M	2	11	27	2	83	0	122	0	150	1
6 A 10,354	15	M	2	4*	399	36	75	22*	114	27	142	22
7 A 10,425	19	M	2	6	142	15	63	0	102	0	130	0
8 A 6,400	23	M	2	6	97	20	63	3	102	3	140	6
9 A 10,423	16	M	2	6	14	1	68	0	107	1	135	2
10 A 10,472	27	M	2	13	49	3	55	0	107	0	135	0
11 A 10,473	17	M	1	13	130	13	60	0	107	3	135	13
12 A 10,475	46	M	1	14	114	0	55	0	94	0	122	0
13 A 10,515	21	M	1	12	100	64	48	1	87	11	115	6

\* Patient treated for two days, treatment withheld for one week due to upper respiratory infection and then repeated for another 2 days.

TABLE 1—(Continued)

Patient No., hospital No.	Age	Sex	Amount of drug administered orally		Number of microfilariae in 60 cu.mm. of blood					Follow-up examinations				
			Mgs. per kilo t.i.d.	Length of Rx	Before Rx	2nd day of Rx	Days after Rx	No. of micro- filariae	Days after Rx	No. of micro- filariae	Days after Rx	No. of micro- filariae	No. of micro- filariae	No. of micro- filariae
14 A 10,591	27	F	1	11	34	0	69	0	108	0	136	0	136	1
15 A 10,596	18	F	1	13	538	0	34	0	73	0	101	0	101	0
16 A 10,608	19	F	0.5	11	10	2	33	1	72	0	100	0	100	0
17 A 10,607	21	F	0.5	11	36	11	33	4	72	1	100	1	100	8
18 A 10,665	22	F	1	5	17	0	23	1	62	4	90	4	90	9
19 A 10,686	26	F	1	6	45	0	20	1	59	0	87	0	87	3
20 A 10,687	18	F	1	6	7	0	20	0	54	0	84	0	84	0
21 A 7,716	33	M	1	3	158	18	15	8	54	17	82	17	82	21
22 A 10,740	19	M	1	3	46	3	10	2	49	0	77	0	77	0
23 A 10,739	15	M	1	3	13	4	10	0	49	0	77	0	77	1
24 A 8,455	21	M	1	5	24	2	8	1			103		103	1
25 A 3,092	33	M	1	5	11	2	8	1	47	1	75	1	75	1
26 A 10,778	20	M	1	5	20	3	8	4	47	7	75	7	75	3

The data presented below show that 1-diethylcarbamyl-4-methylpiperazine hydrochloride, administered orally, causes the rapid disappearance of microfilariae from the blood stream, that no serious toxic manifestations are evoked, and that very possibly the drug also acts on the adult filarial worms.

## METHODS

The twenty-six patients included in this study were all Puerto Ricans, twenty males and six females, ranging in age from 15 to 30 years. Twenty-three of the twenty-six were asymptomatic cases found to have positive night smears for microfilariae during a survey performed by one of us\* at night schools in San Juan, or else were young men rejected from service in the armed forces for the same reason. The three other cases had symptoms suggestive of clinical filariasis, and these also were found to have microfilariae in their peripheral circulation at night.

All patients were hospitalized for treatment. On admission, a complete medical history was taken and they were subjected to rigid physical examination, special attention being given to the lymphatic system. The following laboratory tests were performed on admission: (1) complete blood and differential counts; (2) complete blood chemistry determination; (3) urinalysis; (4) fecal examinations; and (5) Kahn serologic test. The blood chemistry, complete blood count, and urinalysis were repeated every third day during the period of hospitalization.

On the night before therapy was started and on regular occasions during treatment and thereafter, blood smears were taken. The time of bleeding in all was always from 7:45 to 8:30 p.m. The number of microfilariae was estimated in three smears of 20 cu.mm. of blood. The blood films were dehemoglobinized in water, fixed in absolute alcohol and ether (equal parts of each), stained with hematoxylin, and destained with acid alcohol. The microfilariae were counted at a magnification of 100 diameters. The recorded values thus represent the total number of embryos per 60 cu.mm. of blood.

The drug was administered orally, three times daily, before or after meals, or every eight hours. The individual dose varied from 0.5 mg. to 2 mg. per kilogram of body weight and the number of days during which treatment was given varied from three to twenty-one (see TABLE 1). Ten cases were given a daily dosage of 2 mg. per kilogram of body weight, three times a day; two patients received 0.5 mg. per kilogram of body weight, t.i.d. The rest received 1 mg. per kilogram of body weight, t.i.d. The patients were seen and examined daily; any complaints and pertinent physical findings were recorded.

## RESULTS AND COMMENTS

**Reaction to the Drug.** The drug was well tolerated in all instances. Unfavorable reactions due to the drug were never of such a degree as to warrant discontinuation of treatment.

\* J. Oliver-Gonzalez.

The following were the most common reactions encountered, given in their order of frequency, with the number of patients affected; fever (16), headache (12), general malaise (9), lumbar ache (7), anorexia, nausea and vomiting (5), painful nodular swellings (4), aching joints (3), weakness (2), skin manifestations (2), pain in testes (1), shortness of breath (1), and pain in the abdomen (1).

*Fever.* This was usually slight, in eight cases being not over 100°F., in six cases between 100° and 101°, and only in two cases reaching up to 103°F. In almost all patients, the fever started on the second day after initiation of treatment and lasted from one to six days (in only one case), the average duration being 48 hours. One patient, while having fever, complained of some shortness of breath. Febrile cases had an accompanying tachycardia which followed the fever curve. No arrhythmia was noted and there were no changes in the blood pressure.

*Headache.* Slight headache, requiring no medication and disappearing spontaneously within a few hours, was experienced by nine patients during the first or second days of treatment. In three cases, the headache lasted for two days.

*General Malaise.* This occurred in nine cases, in four of which it lasted for only one day, in another four for two days, and only in one case did it last for three days. Only one patient was bed-ridden because of general malaise, which, however, lasted only one day. This case had a high microfilarial count and received the highest dose (2 mg.). All but one of those complaining of general malaise of over one day's duration were receiving the highest doses.

*Lumbar Ache.* Five cases complained of slight lumbar ache, which lasted for one day; two had moderate ache lasting two and three days, respectively. All but two of these cases were receiving the highest dose.

*Anorexia, Nausea, and Vomiting.* All five cases who complained of nausea also had anorexia. These complaints lasted for only one day in three cases, and in the rest for two days. Out of these five cases, three had accompanying vomiting, which, however, was never severe and at the most occurred only twice daily.

*Nodular Swellings.* Four cases developed nodular swellings during treatment, or just after its completion. Two of them had been asymptomatic previously. Case No. A-10423 had received 2 mg. per kilogram of the drug three times a day for six days. On the fifth day of treatment, he complained of slight pain on both testes and over the lower abdomen. Two days later, nodular, moderately tender swellings the size of a small olive were noted along both spermatic cords. There was evidence of an accompanying bilateral funiculitis and some inguinal lymphadenopathy, but without fever. The tenderness and swelling had disappeared almost completely by the third day, and check-up examinations three weeks

later showed only a non-tender, pea-sized nodule in the right cord. Case No. A-10778 complained, on the second day of treatment, of pain at the inner aspect of the left upper arm and by the following day was found to have a hard, nodular, tender swelling at this region with some accompanying axillary lymphadenitis. The swelling had almost completely disappeared within three days.

The other two cases had suffered filarial manifestations prior to start of treatment. Case No. A-10596 had been admitted to another hospital two months previously because of a febrile episode, of ten day's duration, with a tender nodular swelling in the inner aspect of the left upper thigh, diagnosed as an acute attack of filariasis, after smears had been found to be positive for microfilariae. On the fourth day after beginning treatment here, she developed a tender nodular mass about the size of a walnut at the inner aspect of the same thigh just above the site of the previous nodule. The patient had fever up to 103°F. The swelling gradually receded and became less tender until, on discharge twenty days later, it was the size of a small olive and non-tender. Case No. A-8455 had been suffering for over a year from frequent attacks of tender inguinal adenitis and funiculitis accompanied by fever. Biopsy of a nodule from the right spermatic cord was reported as "Hypertrophy and varicosity of lymphatics in spermatic cord with thrombosis and organization, cause undetermined." Microfilariae were found several times in the peripheral blood. Four days after start of treatment, he complained of pain in the right spermatic cord, which appeared tender, thickened, and nodular. This was accompanied by fever. Within two days, the tenderness and swelling had disappeared.

*Aching Joints.* Three patients complained of ache in the shoulders, knees, and elbows. It was mild in two cases and moderately severe in the other, and never lasted over two days.

*Weakness.* Both cases who complained of weakness, lasting two and three days, respectively, were receiving the highest dose of the drug.

*Skin Manifestations.* One patient developed an erythematous rash on the palms of the hands and the soles of the feet, which made its appearance on the third day of treatment and cleared up entirely within three days. This patient was receiving the highest dose. Another patient developed a pruritic papular rash over the antero-lateral aspect of the left leg on the fourth day of treatment, disappearing completely within 24 hours. This same patient complained of soreness of the mouth while having the rash.

*Pain in Testes.* One patient developed pain over the testicular region on the fifth day of treatment, lasting one day, but this was not followed by noticeable swelling or nodules in this region.

*Discussion.* It is apparent that the reactions occurred oftener and were of a more marked degree in the patients who were receiving the

highest dose. Fever and other related complaints first appeared, in most cases, by the second day of treatment and by 48 hours had disappeared spontaneously, never to recur in spite of continuation of treatment. Previous toxicity studies on normal healthy adults had shown that when a high dosage (8 mg. per kg. of weight) was used, the subject complained of headache, general malaise, weakness, joint pains, and anorexia. The other symptoms, that is, fever, tachycardia, tender nodular swellings, etc., we therefore consider to be related to the filaricidal action of the drug, probably developing on a foreign protein or allergic reaction basis.

### **Alterations in the Hematologic Picture:**

*Leucocytosis.* There were no changes in the hematologic picture during or after therapy, except in the leucocytic series. The admission white blood cell count in these cases ranged from four to eleven thousand, the average ranging from five to seven thousand. In all but one case, the drug produced a definite leucocytosis. Usually, this first made its appearance by the second day of treatment and rapidly and progressively reached its peak by the fourth or fifth day. From then on, there was a tendency for the leucocytes to decrease progressively, the descent being at a much slower rate, and twenty or more days later the leucocytic picture still being higher than on admission. The white blood cell count, when at its highest, ranged from five to twenty-four thousand, the average at this time being from nine to eleven thousand. The degree of leucocytosis did not appear to be related to the degree of fever, to the initial microfilarial count, or to the dosage given.

*Eosinophilia.* On admission, twenty-four cases had an eosinophilia of more than 5 per cent, ranging in most of them from 6 to 10 per cent. The lowest eosinophile count was 1 and the highest 32 per cent, the latter being observed in a patient having a massive hookworm infestation. Only three cases failed to show an increase in eosinophiles. Twelve of the patients developed an eosinophilia of over 20 per cent while undergoing treatment. Six of these had over 30 per cent, the two highest figures recorded being 40 and 41 per cent, the last one in a patient having a very high initial microfilarial count (220). The eosinophilia did not appear to be related to the degree of fever, nor to the initial microfilarial count in the other cases. There was, however, a definite relation between the appearance of nodular swellings and eosinophilia, since in the four patients who developed the nodular swellings the initial eosinophile count became three, four, six and eight times higher, respectively.

*Blood Chemistry and Urinalysis.* No changes were recorded in the blood chemistry values or in the urine examinations during treatment or thereafter.

**Effect of the Drug on the Microfilariae.** TABLE 1 shows that in all instances the microfilarial counts dropped markedly within 48 hours

after beginning of treatment. Nine patients (34.6 per cent) had negative blood smears at the end of the 48-hour period, and the other seventeen (65.4 per cent) had their counts markedly reduced.

Thirteen patients (50 per cent) were found to be negative upon subsequent examination made 8 to 83 days after treatment. The rest of the patients had extremely low microfilarial counts, to such an extent as to be definitely suggestive that their complete disappearance would eventually be demonstrated. Only one patient (No. 6—A 10,354) who initially had the second highest filarial count of this series (339) showed a relatively high count of 22 microfilariae on the 75th day after treatment. This patient had been receiving the drug for two days, when he developed a severe upper respiratory infection, for which reason therapy was discontinued. One week later, treatment was resumed and the drug was given for only two days. This is suggestive that, for optimal results, therapy must be given without interruption. The latest examination performed 75 to 160 days after treatment has shown total absence of microfilariae in ten cases (38.5 per cent). All other cases, except two (who had 21 and 22 microfilariae, respectively), still had very low counts. Fifty four per cent of the 13 cases who were treated for extended periods, ranging from 11 to 22 days, were found to be negative for microfilariae at the last examination.

Further analysis of the results obtained in the light of the total dosage employed showed the following results (TABLE 2):

TABLE 2

Total dosage Mg. per kg. wt.	No. of cases treated	Negative at last examination	Percentage
Up to 15	7	1	14.3
16.5 to 33	6	2	33.3
36 to 39	6	2	33.3
42 to 96	4	2	50.0
102 to 132	3	3	100

The data obtained so far seem to indicate that, for optimal therapeutic results, an individual dose of 2 mg. per kg. of body weight should be given 3 times daily for a period of from 2 to 3 weeks.

It is also of considerable interest to note that Hetrazan produced a considerable reduction of the microfilarial count within 48 hours in all cases, regardless of the dosage used, which would seem to indicate that *Wuchereria bancrofti* is extremely sensitive to this drug.

**Effect of the Drug on the Adult Worms.** The appearance in four cases of tender nodular swelling and localized lymphadenitis proximal to the swelling is suggestive of the death of the adult worm. Additional confirmatory evidence is afforded by the marked eosinophilic reaction observed in all four and by the occurrence in three of them of a sharper

rise of fever, which was also of longer duration than in the rest. Though sorely tempted, we refrained from removing any such nodules, as experience has taught that they will be resorbed spontaneously in most cases, while surgical intervention may produce irreparable damage to the lymphatics.<sup>7</sup> We were further restrained by the fear that such intervention would have a poor psychological effect on the volunteer group of patients. Biopsies of similar nodules performed here and by other observers have frequently disclosed the presence in them of dead filarial worms.<sup>8, 9</sup>

For definitive proof of an effect on adult female worms, we have considered it advisable to observe the cases for a total period of at least one year. This is the approximate time necessary for maturation and parturition of worms which might have been inoculated shortly before the start of therapy. Should these patients remain negative for the length of that period, it might then be safely assumed that all adult worms had been destroyed, or that the worms may have become sterile.

## SUMMARY

Twenty-six individuals harboring microfilariae of *Wuchereria bancrofti* in their blood were treated with 1-diethylcarbamyl-4-methylpiperazine hydrochloride (Hetrazan). Twenty-four of the cases were asymptomatic; the other two had clinical pictures of filariasis. The drug was given by mouth, the doses varying from 0.5 mg. to 2 mg. per kg. of body weight, three times daily. All patients were hospitalized and the period of treatment varied from three to twenty-two days.

Although the patients were carefully watched and examined daily, including frequent laboratory determinations, no severe toxic reactions were observed in any of them. Some of the patients, particularly those who received the highest dosage, developed fever and some accompanying symptoms of minor degree. These complaints occurred only during the first forty-eight hours of treatment; from then on, the patients were symptom-free regardless of the duration of therapy. Four cases developed tender nodular swellings at the extremities or spermatic cord, with accompanying lymphadenitis and a higher degree of fever and eosinophilia than the rest.

In all instances, the number of microfilariae was markedly reduced by the second day of treatment. In nine patients (34.6 per cent), the counts were negative by this stage of therapy. Thirteen patients (50 per cent) were found to be negative upon subsequent examination 8 to 83 days after treatment. The latest examination, performed 75 to 160 days after treatment, has shown total absence of microfilariae in ten cases (38 per cent). All other cases, except two, still had very low counts. Fifty per

<sup>7</sup> National Research Council, Division of Medical Sciences. A Manual of Tropical Medicine. Saunders. Philadelphia. 1945.

<sup>8</sup> Kopplisch, E., Personal communication.

<sup>9</sup> King, B. G., Early filariasis diagnosis and clinical findings. A report of 268 cases in American troops. Am. J. Trop. Med. 24: 285-299. 1944.

Wartman, W. B., Lesions of the lymphatic system in early filariasis. Am. J. Trop. Med. 24: 299-313. 1944.



# *Development of the Thyroid Gland*

(SCHEMATIC)



3 mm. embryo—fourth week.  
Thyroid anlage developing as a  
bud from ventral surface of prim-  
itive foregut. Primitive trachea  
“yellow” budding off from  
pharynx.



10 mm. embryo—sixth week.  
Thyroid enlarging, trachea has  
developed into a tube.



15 mm. embryo — seventh week.  
Thyroid has migrated down in  
front of trachea. Thyroglossal  
tract obliterated. Black broken  
line indicates hyoid bone.



Sagittal section to show post-  
uterine condition.

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THE INHIBITION OF MALARIAL RELAPSES BY  
TOXOID OF *CLOSTRIDIUM TETANI*\*

BY

EUSEBIO Y. GARCIA

*Parasitologist and Senior Malariologist,  
Medical Research Clinic, Binan, Laguna, Republic of the Philippines*

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# THE INHIBITION OF MALARIAL RELAPSES BY TOXOID OF *CLOSTRIDIUM TETANI*

## *Introduction*

ALTHOUGH *Clostridium tetani* toxoid has been used as a prophylactic against tetanus for several years, its possible effects on malarial parasites were investigated only recently. This investigation was inspired by an observation of the writer on a mental case of a colored American who was under induced *Plasmodium vivax* malaria treatment at the Florida State Hospital in July, 1946. In the course of the treatment, this American developed gas gangrene of the perineum and buttocks which was later found to be due to *Clostridium welchii*. Surprisingly, the parasitemia which was present before the onset of gangrene disappeared spontaneously on the second day of infection. Subsequent blood examinations were negative for *P. vivax*.

When the writer returned to the Philippines, the possible malaricidal action of exotoxins was put to a test, using at this time toxoid of *Clostridium tetani*, which is a close relative of *Clostridium welchii*. The results of this experiment are gratifying in that another use of tetanus toxoid has been found. This is its anti-relapse activity against the malarial parasites.

These findings are important not only to the public health services and armed forces in the control of tetanus and malaria but also to an academic understanding of the relation between bacterial products and antimalarial activity. The present report records experiments which confirm and seem to explain such activity.

## *Materials and Methods*

Subjects for this study came from naturally infected individuals who are permanent and temporary residents in endemic regions. Only individuals in actual clinical attack were employed in this experiment. Individual clinical history, consisting of duration of residence in endemic regions, when evacuated to non-endemic region, date of first attack while residing in non-endemic region, number of relapses while in endemic region, size of the spleen at the time of consultation, species and parasite count at the time of the attack, was taken carefully before giving the treatment. Subjects were asked to report 24 hours after the initiation of treatment, seven days later, three weeks later, and then once every two weeks thereafter. Parasite counts were taken before and in the subsequent fixed periods, in order to determine the efficacy of the antimalarial drugs under trial on our local strains and at the same time to determine the complete disappearance of parasites in the

peripheral blood during the course of observation. Parasite count represents the number of parasites per 100 leucocytes, and from this the number per cubic millimeter of blood was computed on the basis of the total leucocyte count of the patient when the smear was taken.

Powdered atabrine in 0.20 gm. ampules (Winthrop) and chloroquine (SN 7618; Winthrop) were used in this study. Since *Clostridium welchii* toxoid was not available in our local markets at the time this experiment was contemplated, *Clostridium tetani* toxoid was used. *C. tetani* and *C. welchii* are very similar biologically. They are both spore-forming, anaerobic, and exotoxin-forming, although the toxin of tetanus is more poisonous. The method of treatment employed in all cases was as follows, in their chronological order:

- (1) After preliminary clinical and laboratory examinations, the individual, whether in the chilling, febrile or sweating stage, received in the deltoid muscle a dose of 0.10 gm. atabrine dissolved in 5 cc. of distilled water.

- (2) Four hours after the injection, the patient was advised to take atabrine tablets (0.10 gm. each) three times a day, one or two hours after each meal, for seven consecutive days. Doses for children were calculated according to age. The patient was advised further to come to the clinic the next day and at the end of seven days. To a number of patients who did not like atabrine, chloroquine in a dose of 0.5 gm. daily was given. (Steps Nos. 1 and 2 form the basic treatment.)

- (3) At the end of seven days, blood smears were taken and parasites were counted. In all cases studied, the blood smears (thick and thin) were completely negative at the end of this time. Then 1 cc. of tetanus toxoid was given subcutaneously in the deltoid region. The same dose was given three weeks later. All of the patients were asked to report periodically, once every two weeks, for checking of their blood.

Patients were allowed to resume their previous routine occupations, whether in endemic or non-endemic regions. There was no change in their diet and no precautions were given as to their chance of reinfection, especially to those in the endemic regions. This was done in order to imitate as much as possible what was taking place in nature. Besides, if precautions were given and they were not followed by some, interpretation of the results might be complicated. To forestall errors, therefore, arising from such factors, it was decided to leave the patients as they were before treatment.

With the preceding method as the basic treatment, the subjects were divided into two main groups: those living in endemic regions and those exposed in endemic regions but residing in non-endemic places. Each main group was further classified into primary and secondary attacks (first relapse) with their corresponding species of parasites. Every subgroup had a corresponding control group not receiving tetanus toxoid. Only patients

with clear clinical histories of having primary and secondary attacks, irrespective of the blood findings, were considered. The many patients applying for treatment in the clinic had made possible this selective method.

The criterion used to determine whether or not a patient in endemic regions developed a reinfection after treatment, was the presence or absence of gametocytes in the peripheral blood. This was done by advising the patient under treatment to report at once upon notice of slight malaise within 7 days. If gametocytes were present together with asexual stages, this was taken to mean that the patient still had the original infection; but if asexual stages alone were present, this was indicative of reinfection. A mixed infection occurring later was also indicative of new infection, but not necessarily indicating a relapse of the original infection unless gametocytes of that species were present.

An attempt to determine the protective capacity of tetanus toxoid alone against *P. vivax* malaria, through inoculation of a predetermined number of asexual stages per cubic millimeter from an infected person to a sound individual, failed when volunteers for this purpose could not be found. However, a preliminary experiment along this line, done in naturally infected individuals, indicated roughly that, against patent parasites, tetanus toxoid alone was inadequate to prevent a future relapse. Experimental evidence along this line of investigation is still insufficient to warrant inclusion in this report.

### Results (see also FIGURES 1-4)

The results of this experiment, presented here in three tables, deal specifically with the anti-relapse activity of tetanus toxoid against *P. vivax* and *P. falciparum* infections, by using as base line patients in actual clinical attacks with positive bloods. Reasons for this method of attacking the problem will be touched upon in the discussion in this paper. As stated in the preceding section, no attempts have been made, as yet, to determine the protective capacity of tetanus toxoid alone against malaria, because of difficulties beyond the control of the writer and the possibility that the experiment dealt with in this report might not be a success. Now that the results of this experiment furnish some evidence indicating a more or less positive anti-relapse activity, it becomes an absolute necessity in the future to determine its inherent protective capacity. Hence, this subject will be dealt with in our next report.

TABLE 1 shows the incidence of parasite relapse in *P. vivax* and *P. falciparum* malaria from endemic regions following basic treatment and 2 cc. tetanus toxoid, together with control, within a period of 330 days. It is noted that in group I only *vivax* cases, in both primary and secondary infections (1st relapse), have relapses occurring at the rates of 3.3 per cent and

TABLE 1

Incidence of Parasite Relapse in *P. vivax* and *P. falciparum* Malaria from Endemic Regions Following Basic Treatment and Tetanus Toxoid within a Period of 330 Days

Group	Treatment	Clinical stage	Species	Number of patients treated	Number of patients with parasite relapse, observed for indicated days after treatment												Patients with parasite relapse		Average interval to relapses	
					31 to 60	61 to 90	91 to 120	121 to 150	151 to 180	181 to 210	211 to 240	241 to 270	271 to 300	No.		%				
					to	to	to	to	to	to	to	to	to	No.	%					
I	0.10 gm. atebaine injection on 1st hour; 2.10 gm. atebaine tabs. 3x a day for 7 days; 2 cc. tetanus toxoid in 2 injections at 21-day intervals	Primary	<i>P. vivax</i>	30	0	0	0	0	0	0	0	0	0	1	1	3.3	29	96.7	315	
			<i>P. falciparum</i>	6	0	0	0	0	0	0	0	0	0	0	0	0	6	100	0	
		Secondary	<i>P. vivax</i>	40	0	0	0	0	0	0	0	0	0	0	1	1	2.5	39	97.5	315
			<i>P. falciparum</i>	10	0	0	0	0	0	0	0	0	0	0	0	0	10	100	0	
II	0.10 gm. atebaine injection on 1st hour; 3.5 gm. chloroquine tabs. 2x a day for 7 days; 2 cc. tetanus toxoid in 2 injections at 21-day intervals	Primary	<i>P. vivax</i>	12	0	0	0	0	0	0	0	0	0	2	2	16.6	10	83.4	315	
			<i>P. falciparum</i>	5	0	0	0	0	0	0	0	0	0	0	0	0	5	100	0	
		Secondary	<i>P. vivax</i>	15	0	0	0	0	0	0	0	0	0	0	1	1	6.6	14	93.4	315
			<i>P. falciparum</i>	8	0	0	0	0	0	0	0	0	0	0	0	0	8	100	0	
III (Control)	0.10 gm. atebaine injection on 1st hour; 2.10 gm. atebaine tabs. 3x a day for 7 days	Primary	<i>P. vivax</i>	10	1	5	2	1	0	0	0	0	0	0	9	90	1	10	105	
			<i>P. falciparum</i>	6	0	2	2	1	0	0	0	0	0	5	83	1	17	120		
		Secondary	<i>P. vivax</i>	10	1	3	2	2	1	0	0	0	0	0	9	90	1	10	120	
			<i>P. falciparum</i>	6	1	2	1	1	0	0	0	0	0	5	83	1	17	105		
Total				158	3	12	7	5	1	0	0	0	5	33		125				

TABLE 2  
Incidence of Parasite Relapse in *P. vivax* and *P. falciparum* Malaria from Non-endemic Regions Following Basic Treatment and Tetanus Toxoid within a Period of 330 Days

Group	Treatment	Clinical stage	Species	Number of patients treated	Number of patients with parasite relapse observed for indicated days after treatment												Patients with no parasite relapse		Average interval to relapses
					31-60	61-90	91-120	121-150	151-180	181-210	211-240	241-270	271-300	301-330	No.	%			
I	0.10 gm. atabrine injection on 1st hour; 2.10 gm. atabrine tabs. 3x a day for 7 days; 2 cc. tetanus toxoid in 2 injections at 21-day intervals	Primary	<i>P. vivax</i>	38	0	0	0	0	0	0	0	1	2	1	4	10.5	34	89.5	300
			<i>P. falciparum</i>	12	0	0	0	0	0	0	0	0	0	0	0	0	12	100	0
		Secondary	<i>P. vivax</i>	32	0	0	0	0	0	0	0	0	1	1	2	6	30	94	315
			<i>P. falciparum</i>	7	0	0	0	0	0	0	0	0	0	0	0	0	7	100	0
II	0.10 gm. atabrine injection on 1st hour; 3.5 gm. chloroquine tabs. 2x a day for 7 days; 2 cc. tetanus toxoid in 2 injections at 21-day intervals	Primary	<i>P. vivax</i>	14	0	0	0	0	0	0	0	1	1	0	2	14.3	12	85.7	285
			<i>P. falciparum</i>	7	0	0	0	0	0	0	0	0	0	0	0	0	7	100	0
		Secondary	<i>P. vivax</i>	12	0	0	0	0	0	0	0	0	1	1	2	16.6	10	83.4	315
			<i>P. falciparum</i>	6	0	0	0	0	0	0	0	0	0	0	0	0	6	100	0
III (Control)	0.10 gm. atabrine injection on 1st hour; 2.10 gm. atabrine tabs. 3x a day for 7 days	Primary	<i>P. vivax</i>	10	1	4	3	1	0	0	0	0	0	0	9	90	1	10	105
			<i>P. falciparum</i>	7	1	2	2	1	0	0	0	0	0	0	6	86	1	14	105
		Secondary	<i>P. vivax</i>	10	1	2	3	2	1	0	0	0	0	0	9	90	1	10	120
			<i>P. falciparum</i>	6	0	2	1	2	0	0	0	0	0	0	5	83	1	17	120
Total				161	3	10	9	6	1	0	0	2	5	3	39		122		



2.5 per cent, respectively. Average interval to relapse was 315 days. *Falciparum* cases were all cured. Similarly, in group II, only *vivax* cases, with rates of 16.6 per cent and 6.6 per cent, respectively, relapsed from both primary and secondary infections. Average interval to relapse was 315 days. All *falciparum* cases were also cured. In group III (control), on the other hand, where patients received only the basic treatment without tetanus toxoid, relapses occurred as early as 30 days and as late as 150 days in both *vivax* and *falciparum* cases. Mean average relapse rates of 90 and 83 per cent, respectively, occurred in *vivax* and *falciparum* infections in both clinical stages. Mean average interval to relapses of the two species in both primary and secondary stages was 112.5 days by computation.

TABLE 2 shows the incidence of parasite relapse in *vivax* and *falciparum* malaria from non-endemic regions following basic treatment and tetanus toxoids, together with controls, within a period of 330 days. It is noted that, as in TABLE 1, only *vivax* cases of group I, in both primary and secondary infections, had relapses, with rates of 10.5 and 6 per cent, respectively. Average interval to relapses in both clinical stages was 307.5 days by computation. All *falciparum* cases again were cured. Likewise in group II only *vivax* cases, both the primary and secondary, relapsed with rates of 14.3 and 16.6 per cent, respectively. All *falciparum* cases were cured. Average interval to relapse in both clinical stages was 300 days by computation. In group III (control), patients received only basic treatment without tetanus toxoid, and relapses in *vivax* and *falciparum* cases occurred as early as 30 days and as late as 150 days in both primary and secondary infections. Mean average relapse rates in *vivax* and *falciparum* patients were 90 and 84.5 per cent, respectively, in both clinical stages. Mean average interval to relapse of the two species in both primary and secondary stages was also 112.5 days by computation.

TABLE 3 shows comparison of relapse rates in *vivax* malaria in treated and untreated groups from both endemic and non-endemic regions. The arithmetic mean in the treated primaries and secondaries of groups I and II is 9.51 per cent, while in the untreated or control group it is 90 per cent. These data are not subjected to further statistical treatment because by inspection alone the mean difference is very highly significant. From these figures, therefore, it can be gathered that the absence of parasite relapse from the end of 150 days to 300 days is due to the action of tetanus toxoids.

### Discussion

In this report, parasite relapse in malaria refers to the reappearance of parasites in the peripheral blood following treatment or spontaneous clinical recovery from the primary attack. This is different from febrile relapse in that the latter occurs later and is announced by the symptoms of chills, fe-

TABLE 3  
Comparison of Relapse Rates in *P. vivax* Malaria under  
Basic Toxoid Treatment and Basic Treatment or Control

Group	Clinical stage	Mean average parasite relapse rate	
		Basic toxoid	Control
I	Primary	6.9	90
	Secondary	4.25	90
II	Primary	15.4	90
	Secondary	11.5	90
Arithmetic Mean		9.51	90

NOTE: Square deviation, standard deviation, and probable error of mean in group III (control) is zero. Therefore, mean difference is  $90 - 9.51 = 80.49$ , which, when compared with P. E. of difference, is very highly significant.

ver, and sweating; while the former is discovered only in periodic routine blood examinations. In line with this definition, the present work was done to determine the effect of tetanus toxoids on parasite relapse.

The attack on the problem was based on a working hypothesis that any possible effect of toxoid on the parasites would be a side effect on the immunological mechanism of the body. With this in mind, the experiment was performed on selected subjects who were in actual clinical attacks and whose immunity to malaria was presumed to be practically zero. This is important in the formation of a base line of immunity from which the effect of the toxoid, if there is any, can be measured. This base line, however, is conditioned not only by the actual clinical attack but also by the number of such attacks, species of the parasites, and environment. Such being the case, the subjects under study were classified on the basis of primary and secondary infections, species of the parasites, and environment (whether in endemic or non-endemic regions). With this method, in which the base line of immunity is reduced to practically zero or to an estimated quantity, it should be easy to single out the effect of the toxoid after the elimination of the factors of clinical stages, species of the parasites, and environment.

To eliminate the influence of clinical stage, it is necessary that immunity produced by crisis after either primary or secondary attack should be terminated or reduced to an insignificant level by drug action in the shortest possible time. The time factor involved deserves important consideration, because any prolongation beyond the specified period will modify the degree of immunity developed after each crisis, and this will complicate the interpretation of results. To satisfy this requirement, therefore, a strong anti-malarial drug with proven efficacy should be used. For this purpose, the

basic treatment consisted of one injection of 0.10 gm. atebriane, followed five hours later by (1) a series of doses totaling 2.10 gm. of atebriane (one 0.1 gm. tablet at five-hour intervals for seven days) or (2) a total of 3.5 gm. of chloroquine tablets given at the same intervals for the same length of time. Previous experience had shown that this treatment can sterilize the blood of the patient from all stages, whether asexual or gametocytes, of whatever species involved, at the end of 7 days. The initial injection of atebriane will produce a plasma level of the drug sufficient to kill 50 per cent of the erythrocytic parasites in 24 hours. This level can be maintained by subsequent doses of this or another drug of the same kind by mouth at 5-hour intervals for seven days. The first injection is the so-called knockout dose followed by light punches which are the tablets given by mouth. Group III, which serves as control, received only this basic treatment to suppress the acute clinical attack. Incidentally, it can be stated definitely that this basic treatment has a distinct advantage over other methods of treatment in that, in chronic relapsing malaria, it can destroy almost 99 per cent of the erythrocytic asexual stages in 20 hours and all sexual stages in 7 days.

As a general rule, immunity is increased with succeeding attacks and is greater in patients in endemic than in non-endemic regions. This is shown in group I, TABLE 1, in which the difference in relapse rates for *P. vivax* malaria is insignificant, while in group I, TABLE 2, the difference in relapse rate between primary and secondary *vivax* malaria may be due to the relatively slight immunity produced by the first relapse, or to the possibility that the interval between the primary and first relapse is too short for the forces of immunity to develop to an appreciable extent. The fact that the relapse rate in primary *vivax* malaria, group I, under non-endemic region, is significantly higher than the secondary under the same region, may be explained by the general rule that, under the same environment, there is more immunity conferred by repeated attacks than by one attack. Immunity decreases proportionately to the duration of stay in non-endemic regions and is easily terminated by any extrinsic or intrinsic inimical factors. There is evidence that these differences in relapse rates, dependent on clinical stage and environment, have not affected the base line of immunity during the course of the experiment. This is shown in the average interval to relapses in *vivax* malaria, which is practically the same in two different groups under their respective different regions.

On the other hand, the influence of tetanus toxoid on relapse rates differs with the species of parasites. This is shown in TABLES 1 and 2, in which, irrespective of clinical stages and regions, the relapse rate in *P. falciparum* malaria is zero while that of *P. vivax* malaria is reduced only to a low level. This result can be taken to mean that tetanus toxoid plus the basic treatment can produce cures in 100 per cent of the *falciparum* patients and in 90–

95 per cent of the *vivax* patients. Obviously, *falciparum* malaria is more susceptible to this treatment.

After discussion of the different factors which might influence the anti-relapse effect of the tetanus toxoid, it is appropriate to consider its probable manner and site of action. It was assumed, at the beginning of this discussion, that tetanus toxoid, if it has any action, must exert a side effect on the immunological mechanisms of the body, quite different from its specific action against *Clostridium tetani*. Be this as it may, its action must be non-specific with reference to malaria, but may be direct on the forces of immunity of the body. In this case, then, it is the reticulo-endothelial system that will be affected primarily by the toxoids. These forces of immunity, as Taliaferro and Mulligan (1937) have demonstrated, are purely cellular in origin and, when stimulated or enhanced as in this case by tetanus toxoid, the cells of the reticulo-endothelial system can kill the parasites by intense phagocytosis. In other words, tetanus toxoid serves as a mere stimulant in the operation of this intricate system of immunological mechanisms. According to Kolmer (1945), defense mechanisms may "cripple or injure the proliferative activities of microorganisms, followed by their final destruction or removal from the tissues by phagocytosis or other immunological mechanisms." This is true for both natural and acquired immunity; although action of tetanus toxoid is non-specific to malaria, it may stimulate both forces of immunity to operate against the parasite. Throughout the course of the experiment, therefore, these two forces of immunity, which had been reduced practically to zero before the administration of tetanus toxoid, were probably revived and reinforced, so much so that they were able to prevent the appearance of the parasites in the peripheral blood in 90 to 95 per cent of *P. vivax* malaria and completely destroy them in *falciparum* malaria within a span of 300 days. This explanation is in agreement with the statement of Boyd (1944) that "the ultimate extinction of a malarial infection probably is more attributable to the activation of the body's immunological mechanism than to the administration of drugs already known."

From what has been found and already discussed, there is no doubt that tetanus toxoid exerts an anti-relapse effect in malaria. But it is uncertain what stage of the parasite is susceptible to toxoid action. This can be understood easily if we recall that malariologists are of the opinion that exo-erythrocytic schizogony is the fundamental cause of relapse in malaria. This special stage of the parasite has a special tendency to develop in the reticulo-endothelial cells, including the wandering macrophages and some fixed endothelial cells of the peripheral blood vessels. Since tetanus toxoid is an exotoxin in peptone and intended for active immunization against tetanus and in this case presumably did not have a chance to act on the erythrocytic stages because they were destroyed by the basic treatment, and

since the apparent remaining stage of the parasite is the exo-erythrocytic one in which the parasites are hiding in the reticulo-endothelial cells, it seems easy to predict that the forces of immunity stimulated by the toxoid have been directed to their secret abodes. What happens, probably, is that either the toxoid sensitizes the reticulo-endothelial cells to active phagocytosis or the parasites which have gained a foothold in the cells are inhibited in their growth, resulting in their destruction after a certain time. This harnessing of the reticulo-endothelial system for therapeutic usefulness finds a similarity in the principle involved in the method of Bogomolets (cited by Leake, 1946) in influencing the reticulo-endothelial system to fight disease.

The sensitivity of the reticulo-endothelial system to any immunizing substance is well recognized. It can be inhibited or stimulated by this substance, depending upon dosage. This is the reason why, in giving tetanus toxoids to all patients under study, doses not exceeding 1 cc. per injection and at intervals of 21 days were followed. Higher doses will destroy the efficiency of the reticulo-endothelial system as in peptone shock.

#### *Summary and Conclusions*

A total of 319 patients classified by clinical stages, regions and species of malarial parasites have been tested for the anti-relapse activity of tetanus toxoid. The basic treatment consisting of one injection of 0.10 gm. atebine followed by either a total of 2.10 gm. of atebine tablets by mouth at five-hour intervals for 7 days, or by a total of 3.5 gm. of chloroquine tablets by mouth at the same interval and duration, can kill almost 99 per cent of the asexual stages in the peripheral blood at the end of 20 hours and can sterilize the blood from both asexual and sexual stages at the end of 7 days. This treatment results in relapses at a rate of 90 per cent in both *P. vivax* and *P. falciparum* malaria, at an average interval of 115 days. If, however, this basic treatment is followed by injections of tetanus toxoid, relapse rate can be reduced to a mean of 9.5 per cent in both *vivax* and *falciparum* malaria, and an average interval to relapses of 315 days. The mean difference between the relapse rates following basic treatment alone, and combination with tetanus toxoid, is highly significant. This indicates that tetanus toxoid action has something to do with the absence of relapses between 150 and 315 days after treatment.

In so far as our local strains of *vivax* and *falciparum* malaria are concerned, the efficacy of atebine and chloroquine is practically the same on the basis of the average interval to relapses after treatment. Their average interval to relapses in both *vivax* and *falciparum* patients is 105 days or three and one-half months. With tetanus toxoid, this can be increased to 315 days, which shows that the anti-relapse activity of the combined basic and tetanus toxoid treatment is three times that of atebine or chloroquine.

The probable manner and site of action of tetanus toxoid with respect to the parasite are discussed on the basis of the stimulation afforded to the forces of immunity of the body to act probably against the resistant stage of the parasite which remains in hiding in the reticulo-endothelial cells. This method of utilizing a bacterial toxin to fight another disease through the stimulation of the reticulo-endothelial system finds a similarity in the principle involved in Bogomolets's method of influencing the reticulo-endothelial system for therapeutic usefulness.

These results probably represent the first successful attempts to inhibit multiplication of protozoan parasites by stimulation of immune mechanisms through a bacterial product.

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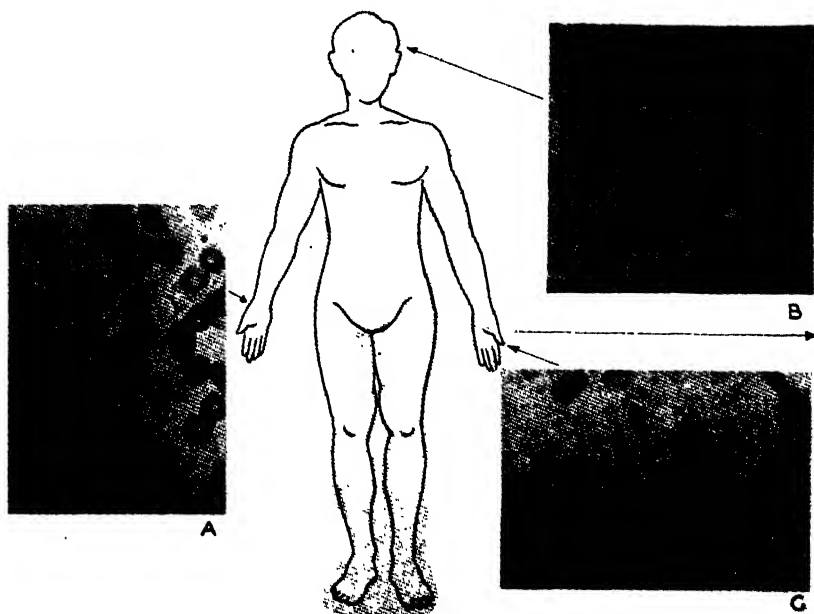


FIGURE 1. Patient with actual clinical attack of malaria.

- A: Peripheral blood smear with a young schizont and ring forms of *Plasmodium vivax*. (450X)  
B: Brain smear with exo-erythrocytic schizonts in one capillary and various asexual stages of *Plasmodium falciparum* in another. (450X)  
C: Thick smear of peripheral blood with various asexual stages of *Plasmodium vivax*. (450X)

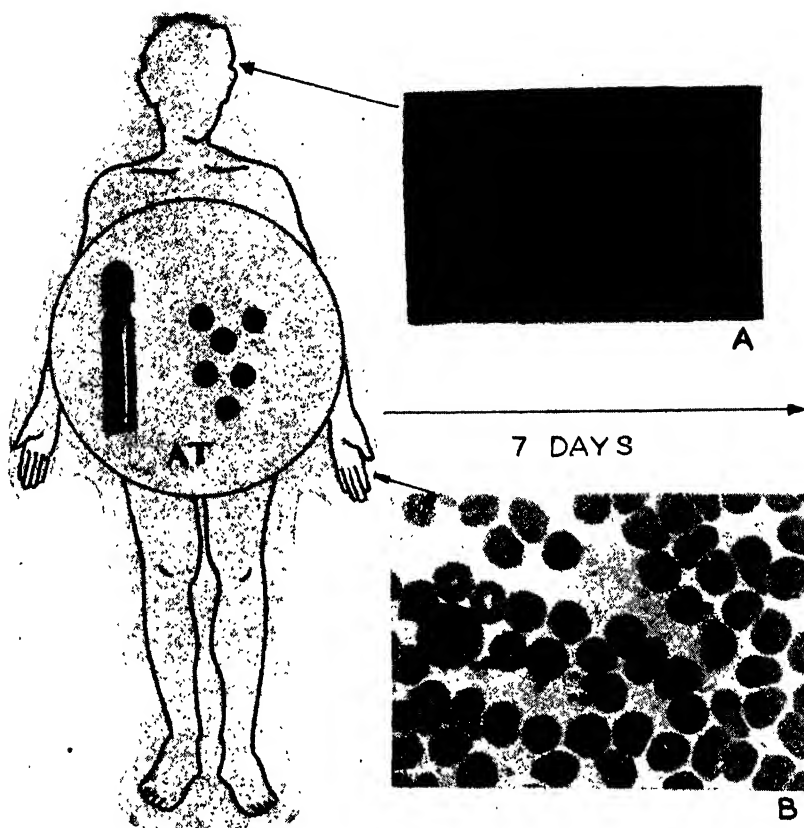


FIGURE 2. Same patient as in FIGURE 1, after treatment with atebrine (AT) for seven days.

*A*: Brain smear with only exo-erythrocytic schizonts remaining. (650X)

*B*: Thin smear of peripheral blood, negative for malarial parasites. (650X)

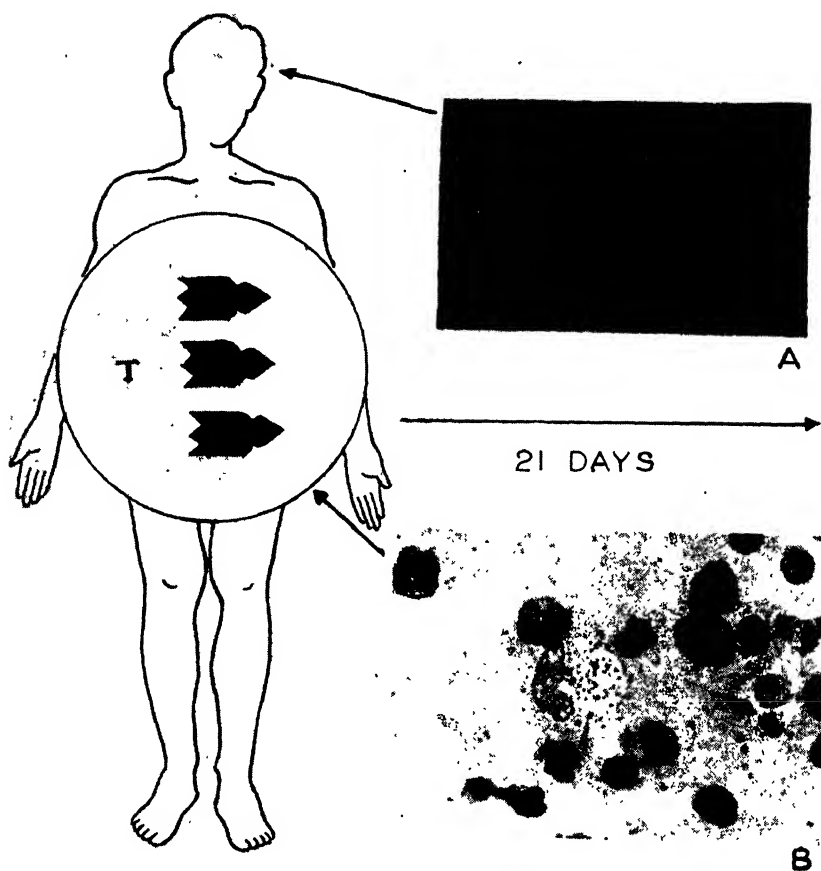


FIGURE 3. Same patient as in FIGURE 2, after receiving one booster shot of tetanus toxoid. Toxoid (T) either attacks the exo-erythrocytic schizonts and/or sensitizes the leucocytes to active phagocytosis.

A: Brain smear with only exo-erythrocytic schizonts remaining. (650X)

B: Splenic smear with one large mononuclear containing four erythrocytic schizonts. (650X)



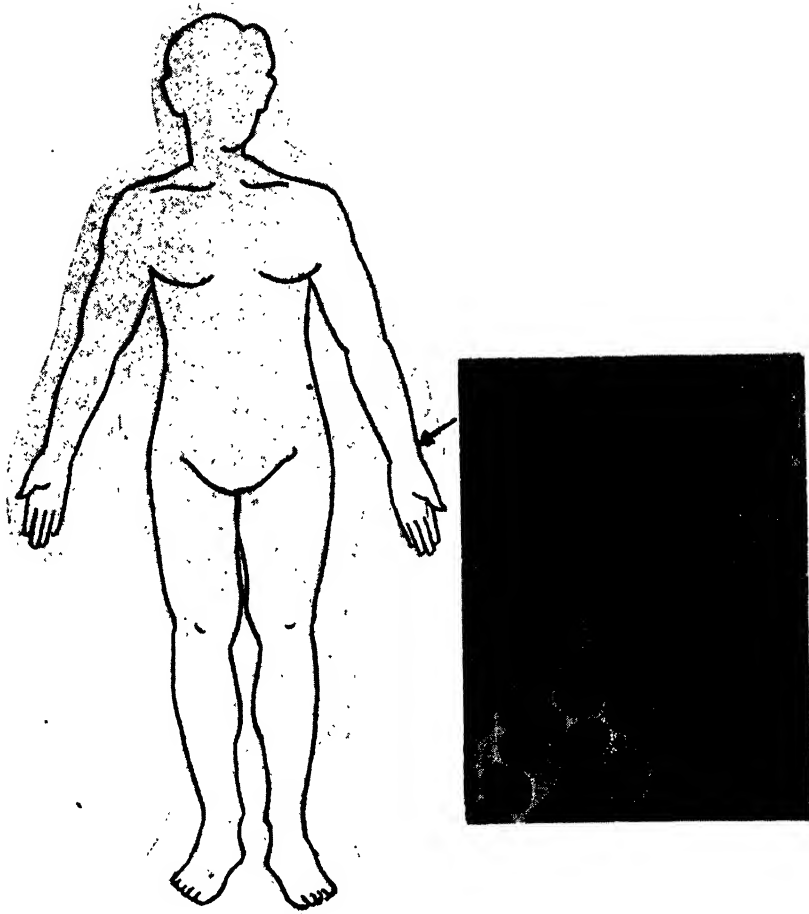


FIGURE 4. Same patient as in FIGURE 3, 21 days after receiving the first booster shot of tetanus toxoid. Exo-erythrocytic schizonts and erythrocytic schizonts remaining in hiding in various internal organs have probably been destroyed. Photograph shows thin peripheral blood smear, negative for malarial parasites. (650X)

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**TELEOLOGICAL MECHANISMS\***

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## FOREWORD

By LAWRENCE K. FRANK

*Caroline Zachry Institute of Human Development, New York, N. Y.*

THE title of this conference—*Teleological Mechanisms*—may be somewhat perplexing or difficult to accept to some who are encountering it for the first time. Hence, a brief statement about this concept and its origin may be helpful in finding the desired orientation.

In looking back through the history of ideas, we can recall that scientific endeavor has had a long and often bitter struggle to free itself from the animistic tradition in our culture. It is enough to refer to the mind-body-soul conception in religion and philosophy and the persistence of psychic entities and vitalistic assumptions in biology and psychology.

The concepts of purposive behavior and teleology have long been associated with a mysterious, self-perfecting or goal-seeking capacity or final cause, directive in all events, usually of a superhuman or supernatural origin. Thus, man has been invested with a soul that guided all his conduct, while non-human events have been viewed as controlled by spirits or, later, by special forces or Maxwellian demons.

To move forward to the study of events, scientific thinking had to reject these beliefs in purpose and these concepts of teleological operations for a strictly mechanistic and deterministic view of nature. A major step in this direction was taken by Galileo, who substituted for the Aristotelian *vis a tergo* the conception of inertia and thereby laid the foundation for particle physics and astronomy.

With the new concepts and the more precise methods of investigation and measurement, science made astonishing advances, progressively revealing the order of events without assuming any goal-seeking or purposive striving—teleological process—in nature. This mechanistic conception became firmly established with the demonstration that the universe was based on the operation of anonymous particles moving at random, in a disorderly fashion, giving rise, by their multiplicity, to order and regularity of a statistical nature, as in classical physics and gas laws.

The unchallenged success of these concepts and methods in physics and astronomy and, later, in chemistry, gave biology and psychology their major orientation. These disciplines approached problems as essentially similar to physics, as problems of the relation between two variables, to be isolated by a progressive fractionation of organisms into ever smaller parts and the statistical manipulation of data to reveal these relations as well as the mechanisms and factors.

This approach to problems of organisms was reinforced by the analytic preoccupation of the western European culture and languages. The

basic assumptions of our traditions and the persistent implications of the language we use almost compel us to approach everything we study as composed of separate, discrete parts or factors which we must try to isolate and identify as potent causes.\* Hence, we derive our preoccupation with the study of the relation of two variables, to be found by abstracting parts from organisms, as in laboratory preparations. We thereby punctuate the order of events more or less arbitrarily, according to our preconceptions and methodological necessities, and then assume that these conceptual mechanisms are the basic processes of nature. As P. W. Bridgman pointed out some years ago, if nature appears to be mathematical, it may be due to our insistence upon asking questions that we can and will answer only in mathematical terms.

Again, the success of these methods has been astonishing, revealing the ever finer structural, functional, and behavioral aspects of organisms.† However, within recent years, there has been a growing perplexity in biology and psychology. As they have pursued this analytic method and disclosed ever more subtle and elusive factors and mechanisms, it seemed to carry them further and further away from the organisms and the personalities they were trying to understand.<sup>1</sup> The inconsistencies and conflicts of theory, as usually happens, started a critical examination of basic assumptions and the organizing conceptions and methodologies.

Thus, we are witnessing today a search for new approaches, for new and more comprehensive concepts and for methods capable of dealing with the larger wholes of organisms and personalities. The term *organism-as-a-whole* has been used, along with *holism*, *synholic*, and similar expressions designed to recognize the patterned, organized structure-function activities, internally and overtly, of living organisms. New methods are being offered for handling a variety of data from the same organism and revealing the systematic interrelationships among many variables or dimensions within the same organism, as well as between the organism and its environment. Emphasis is increasingly being placed upon the role of past experience in modifying organic functions, feelings, and behavior, e.g., the psychosomatic conception in medicine.<sup>2</sup> There also has been growing recognition of the patterning of perception, as in Gestalt psychology and projective methods, of learning other than by trial and error, and a renewed interest in the striving for goals as shown by organisms and individual personalities.<sup>3</sup>

\* Indeed, our language is a "verbal tissue slice" which abstracts the living process from time and space and immobilizes it for discussion.

† We are shifting our focus of interest from static entities to dynamic processes and the order of events as seen in a context or field where there are interreactions and circular processes in operation.

<sup>1</sup> Cf. NORTHROP, F. S. C. Entities and organization in current biological thinking. *Sigma Xi Quart.* 21: 1. 1933. "Ancient biology provided for form with a resulting rigid fixity of types, at the expense of entities and the fluidity of species; modern biological theory provides for the non-fixity of types and the presence of physicochemical and genetical entities, at the expense of organization."

<sup>2</sup> See Ann. N. Y. Acad. Sci. 44 (6): 339-624. 1943.

<sup>3</sup> A clear statement of this appears in: WALTER B. CANNON. *The Way of an Investigator*. Chapter X, "Some Working Principles." Norton. New York. 1945.

The concept of teleological mechanisms, however it may be expressed in different terms, may be viewed as an attempt to escape from these older mechanistic formulations that now appear inadequate, and to provide new and more fruitful conceptions and more effective methodologies for studying self-regulating processes, self-orientating systems and organisms, and self-directing personalities. But these new concepts carry no psychic or vitalistic assumptions, nor do they imply that any mysterious, supernatural powers or psychic forces or final causes are operating the system or guiding the organism-personality. The idea of purposive behavior is not a regressive movement to an earlier stage in the history of ideas, but a forward movement toward a more effective conception of the problems we face today. We are moving toward a conception of a "natural teleology," as Woodbridge suggested in 1911.<sup>4</sup>\*

Thus, the terms *feedback*, *servomechanisms*, *circular systems*, and *circular processes* may be viewed as different but equivalent expressions of much the same basic conception. The idea of self-regulation, with goal-seeking behavior, becomes applicable in the laboratory, in the clinic, especially for study of personality, as well as in the field for study of social orders and cultures. Purposive behavior recognizes the life history and the context, the environmental "field" in which all behavior occurs, thereby revealing events in a time perspective.<sup>5</sup>

It is somewhat ironic that, in order to bring into biology and psychology the concept of purposive behavior and teleological mechanisms, we have leaned so heavily upon the models of man-made machines and artificial systems, such as computing machines, guided missiles, and other complicated electronic devices. We have accepted these artifacts as models or as clues to an understanding of the living organisms provided by nature, learning to see organisms in terms of what man himself has first fabricated. But this is a recurrent process in the history of ideas. The principle of feedback and servomechanisms, which is used as a basic concept in most of the papers of this monograph, is illustrated for general reference in FIGURE 1.

As the conference program indicates, a number of papers on a wide array of problems and diversified materials will be presented, thereby revealing the larger significance of this concept of teleological mechanisms and illustrating its service in different fields of scientific endeavor. Since these several papers will offer interesting and probably provocative materials for discussion, it may be appropriate to point out at the beginning what may be overlooked or ignored in our preoccupation with the specific problems and methodological questions they may raise. We

<sup>4</sup> WOODBRIDGE, F. J. E. *Nature and Mind* (Chapter on Natural Teleology: 113 *et seq.*). Columbia University Press. New York. 1939.

\* Thus, we are beginning to recognize that, during the prolonged period of geological time, a variety of organized, patterned processes have evolved which can be understood only as multi-dimensional circular systems or processes, operating as dynamic, not static, equilibria. Accordingly, the concepts and methods applicable to the random activities of disorderly molecules, as in a gas, are not competent to deal with organisms and personalities.

<sup>5</sup> Cf. FRANK, LAWRENCE K. Time perspectives. *J. Soc. Philos.* 4 (4). 1939.

should not miss, or fail to recognize, the significance and larger meaning of this conference, held at this time.

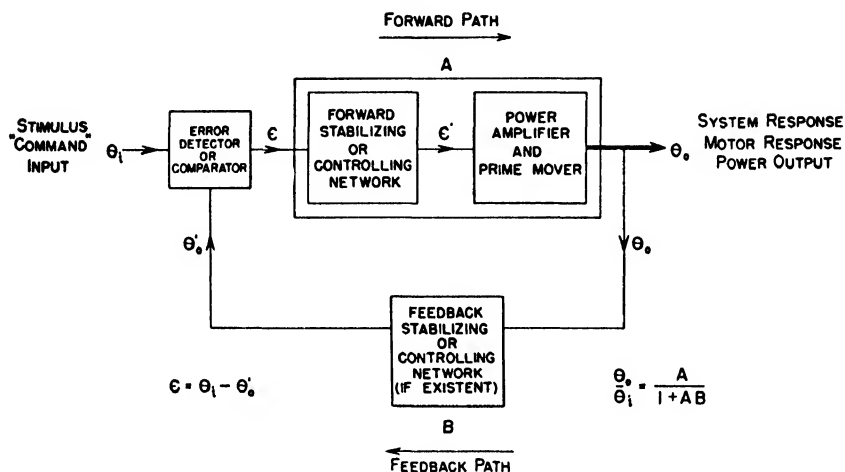


FIGURE 1. An example of a simple feedback system commonly occurring in nature and in certain engineering applications, with basic elements delineated. (Courtesy of Mr. DONALD HERR, Allen-Bradley Co., Milwaukee, Wisconsin. See also his contribution on pp. 274-276.)

It is suggested that we look at this conference as an important, perhaps a major, step toward the new climate of opinion now emerging in scientific, philosophical, and even artistic activities. We are not only witnessing, but, by these meetings and discussions, actively participating in creating this new climate of opinion. We are formulating and critically examining the new concepts and methodologies, not only for obtaining data but for ordering and interpreting them and revealing their interrelationships. We are establishing new criteria of credibility with which to test the validity of these new findings, as well as to elicit new and hitherto unsuspected relationships from older findings. We are, in brief, constructing a new conceptual frame of reference for scientific investigation in the life sciences.

As I see it, we are engaged, today, in one of the major transitions or upheavals in the history of ideas, as we recognize that many of our older ideas and assumptions are now obsolescent and strive to develop a new frame of reference to give us a clearer and more comprehensive understanding of the basic processes underlying all events.

In this transition, many of our long-cherished convictions and expectations must be revised—some to be wholly discarded as archaic and replaced by others more consonant with our new insights and a larger conceptual grasp of the dynamics of events, especially in the life sciences.

In this, we are facing the inescapable and far-reaching implications of what began about the year 1900 in physics. As Einstein and Infeld remarked in *The Evolution of Physics* (page 259):

"It needed great scientific imagination to realize that it is not the charges nor the particles, but the *field in the space between* charges and the particles, which is essential for the description of physical events. . . . The theory of relativity arises from the field problem. . . . The contradictions and inconsistencies of the old theories force us to ascribe new properties to the time-space continuum, to the scene of all events in our physical world."

Whatever may be the mathematical expressions of quantum physics, of time-space, of relativity and field theory, these newer conceptions are compelling us to alter many of our long-cherished assumptions and to reorganize our basic conceptual formulations in the life sciences.

We are, if I do not misinterpret these developments, called upon to recognize that the universe presents at least two major aspects or problems: events exhibit not only statistical order and regularity arising from the *convergence* of many disorderly, anonymous, atomistic particles, as so clearly demonstrated by classical physics; events also occur as *divergent*, often identifiable events, in patterned, dynamic processes, on successive levels of complexity and multi-dimensional interrelationships, as exhibited by organisms.

Irving Langmuir has pointed out<sup>6</sup>:

"We must recognize two types of natural phenomena. *First*, that in which the behavior of the system can be determined from the average behavior of its component parts and, *second*, those in which a single discontinuous event (which may depend upon a single quantum change) becomes magnified in its effect so that the behavior of the whole aggregate does depend upon something that started from a small beginning. The first class of phenomena I want to call *convergent* phenomena, because all the fluctuating details of the individual atoms average out, giving a result that converges to a definite state. The second class we call *divergent* phenomena, where from a small beginning increasingly large effects are produced. In general then we may say that classical physics applies satisfactorily to convergent phenomena and that they conform well to the older ideas of cause and effect. The divergent phenomena on the other hand can best be understood on the basis of the quantum theory of modern physics."\*

The underlying conception of quantum physics implies, beneath disorderly molecular events, a patterned interaction on the subatomic level—the emission of energy as an individual quantum for which there must be an absorber or recipient, an elemental feedback or circular process.

Recently, Schroedinger has offered a clue to the hitherto unresolved problem of heredity in the operation of aperiodic solids which operate, *not* according to the older principles of convergent, statistically regular events, but according to the quantum principle of divergent events.<sup>7</sup>

The principle of indetermination—that we cannot simultaneously measure both the position and velocity of a particle—implies more than a methodological limitation. It indicates a wider and more fundamental situation in biology, where we must recognize that the dynamic processes which we should like to study cannot be isolated by the investigator from the organic field in which they operate, without sacrificing much of what should be observed and measured. That biologists did not enunciate this broader principle of indetermination earlier, is indicative of their reliance

<sup>6</sup> Science 97: 1-7. 1943.      \* Excerpt from pp. 3-4.

<sup>7</sup> SCHROEDINGER, ERWIN. *What is Life?* Macmillan Co. New York. 1946.



upon the traditional analytic procedures and the older concepts of causation which led them to measure ever more precisely the *products*, while neglecting the dynamic *process* producing them.<sup>8</sup>

It may be of interest, here, to recall what a distinguished biologist said some sixty-odd years ago about the relation of biology to physics, the nineteenth-century physics of his day, which has been so profoundly modified in the twentieth century:

"Dr. Haldane holds that to the enlightened biologist a living organism does not present a problem for analysis; it is, *qua* organism, axiomatic. Its essential attributes are axiomatic; heredity, for example, is for biology not a problem but an axiom. 'The problem of physiology is not to obtain piecemeal physico-explanation of physiological process,' (I quote from the 1885 address) 'but to discover by observation and experiment the relatedness to one another of all the details of structure and activity in each organism as expressions of its nature as one organism. . . . That a meeting-point between biology and physical science may at some time be found there is no reason for doubting. But we may confidently predict that if that meeting-point is found, and one of the two sciences is swallowed up, that one will *not* be biology.'"<sup>9</sup>

It is becoming clear, as we recognize time-space and the multi-dimensionality of all organisms, that we must accept what I have called *biological relativity*; namely, that all our measurements are relative and must be ordered to the organic field in which they are observed. Moreover, the older rigid dichotomy of structure *versus* function is becoming obsolete as we realize that, while we can measure an organism spatially or temporally, these are but two dimensions of a time-space complex which is transforming, releasing, and absorbing energy in what we call functional processes.<sup>10</sup> The use of "tagged" atoms and isotopes is showing that the seemingly solid, stable organism is in a continual flux, as all its constituents are being replaced at different rates, although the identified organic pattern or configuration persists relatively unchanged.<sup>11</sup>

In these many and far-reaching transitions and reorientations, we suffer a lack not only of conceptual clarity but also of terminological equivalents for the more recently developed understandings. We have no adequate words, especially verbs, for many processes, especially self-regulating processes, like Cannon's homeostasis and the multi-dimensional relationships they exhibit.

Research in biology and social sciences, conceived as a search for cause-and-effect relations, assumes that living organisms exhibit only the elementary forms of statistical order and regularity as in particle physics. Moreover, the continued use of the older cause-and-effect formula, implying a potent "cause" operating upon a passive something to produce

<sup>8</sup> Cf. FRANK, LAWRENCE K. Gerontology. *J. Gerontol.* 1 (1): 1-11.

<sup>9</sup> From: HOPKINS, SIR FREDERICK GOWLAND. Some chemical aspects of life. *Science* 78 (2020): 227. 1933.

<sup>10</sup> Cf. FRANK, LAWRENCE K. Structure, function and behavior. *J. Phil. Sci.* 2 (2). 1935; also, Man's multi-dimensional environments. *Scient. Monthly* 56: 244-257. 1943; also, Research in orthopsychiatry. *Am. J. Orthopsychiat.* 13 (2). 1943.

<sup>11</sup> SCHOENHEIMER, R. *The Dynamic State of Body Constituents*. Harvard Univ. Press. Cambridge. 1942; also, J. G. HAMILTON. Applications of radioactive tracers to biology and medicine. *J. Appl. Phys.* 12: 440. 1941.

the effect, obstructs our efforts to understand the essential circular processes of action, reaction, and interaction, taking place in the "field" of intra- or interorganic events. The causal concept in biology (or stimulus-response in psychology) ignores the participation of the organism-personality, being acted upon by the so-called "cause" or "stimulus," and continues the animistic conception of some mysterious power or force responsible for events.

This lack of adequate words compels us to continue to speak of "parts," of "wholes," and of "organization," viewed as entities or factors or powers and super-entities. Frequently, a writer, struggling to describe the dynamic process of interrelationships within a "field," will use the older terms which deny or contradict the very "field" conditions he wishes to describe, as in experimental embryology.

Accepted mathematical operations may perpetuate many "atomistic" and linear implications and assumptions, derived from an earlier climate of opinion or from particle physics, which are not consonant with the circular processes and organic "fields" being studied. Hence, many mathematical attempts to order the data of organisms and living systems betray the investigator who trustingly relies upon statistics and mathematics to "solve" his problem. Mathematics, like other methodologies and instruments, is only the "servant of ideas" (Carrel, 1931), and, no matter how rigorous the mathematical treatment of data, will always be guided by underlying assumptions and organizing conceptions.

As Einstein has said<sup>12</sup>:

"There is no inductive method which could lead to the fundamental concepts of physics. Failure to understand this fact constituted the basic philosophical error of so many investigators of the nineteenth century. . . . We now realize with special clarity, how much in error are those theorists who believe that theory comes inductively from experience. . . ."

There is an urgent need for new concepts in the social sciences to replace the anachronistic assumptions inherited from the eighteenth century. With few notable exceptions, economists, political scientists, and sociologists are still using the Newtonian conception of a superhuman, self-equilibrating "system" or mechanism, operated by large-scale "forces" acting at a distance, not unlike gravitation and the ether of classical physics. They then impute to the individual the motives and desires which they derive deductively from statistical studies of group activities, as did particle physics, ignoring what the study of individual personalities has revealed about human behavior.

When the social sciences accept these newer conceptions, especially of the "field," and learn to think in terms of circular processes, they will probably make amazing advances. They will find it fruitful to recognize that the regularities of social life arise from the social-culture patterns and institutional practices into which the individual's activities are channeled. They will then realize that the dynamics of social life arise from individual actions, reactions and interactions, not from the mythi-

<sup>12</sup> EINSTEIN, A. *Physics and reality*. J. Franklin Inst. 222: 349-382.

cal "forces" which they assume operate and control the social "system."

Already, the fruitfulness of the newer approach has been shown in the *psychocultural approach* which has begun to illuminate the dual aspects of social-cultural regularities and of highly individualized personality activities and patterned expressions.<sup>13</sup>

Because we are all in different stages of transition from the old to the new, each of us will approach this conference with the preconceptions and methodological bias of our several disciplines. Each will react differently to the several papers that will be presented. Some we shall eagerly welcome, accepting the newer formulations and procedures, while rejecting others that conflict with older ideas which we loyally cherish although they are no longer congruous with the new ones we have accepted.

As Proust reminded us in his novel, *Remembrance of Things Past*, "Each one can find lucidity only in those ideas which are in the same state of confusion as his own." We shall do well to remember this in the discussion to follow when, if the conference is to be fruitful, we must all make an effort to share each other's confusions. Also, we must try to communicate on a level of generality essential to a meeting of representatives of so many different disciplines. May I recall a statement that seems peculiarly appropriate on this occasion, namely, the penetrating remark of Chancellor Kemp Smith of Edinburgh University in his inaugural address some thirty years ago: "The history of human intelligence is a record, not so much of the progressive discovery of truth, as of our gradual emancipation from error."

This conference may help us to recognize more clearly some of these historic errors and now obsolete ideas, as well as to clarify the new ways of thinking and investigating which we are privileged today to develop. I hope that these preliminary observations will not, however, divert attention from consideration of the several papers to be presented by the various authors. It is highly appropriate that the first will be one by Dr. Wiener, since it was the paper on *Behavior, Purpose and Teleology*, by Rosenblueth, Wiener, and Bigelow<sup>14</sup> which largely initiated these discussions of teleological mechanisms.

<sup>13</sup> Cf. FRANK, LAWRENCE K. *Society as the Patient*. Rutgers University Press. New Brunswick, N. J. 1948.

<sup>14</sup> J. Philos. Sci. 1943.

# TIME, COMMUNICATION, AND THE NERVOUS SYSTEM

By NORBERT WIENER

*Department of Mathematics, Massachusetts Institute of Technology,  
Cambridge, Massachusetts*

IT has long been recognized that no analysis of a natural science, whether it be physics or biology, is complete unless we possess a proper analysis of its appropriate time-concept.

We observe a temporal sequence of events, and our experiments are attempts to reproduce at various times that which we have observed at one particular time. Therefore, all the improvements and modifications which have been made in the theory of time itself are relevant in the study of all the sciences.

There are, however, certain very important refinements of our idea of time which enter, to a rather slight extent, into our study of events on the usual scale of time and velocities. Chief among these are those ideas which are associated with the name of Einstein. Not only is the Einstein gravitational theory, very probably, still far from its ultimate form, but even the Einsteinian special relativity concerns itself largely with effects that become prominent only at very high velocities. These effects, it is true, are important in the study of specific phenomena, biological as well as physical, but there is still a considerable range of phenomena where they appear as small modifications of the theory rather than as necessary notions. Moreover, the relation between the time of relativity and the time of quantum theory has only recently approached any settlement, and this is still *sub judice*.

We shall, therefore, not consider relativistic time in this paper. Neither shall we consider theories of time which give it an atomic rather than a continuously flowing nature. It is possible that such notions may play a decisive part in nuclear physics, but their scale is so different from that of the phenomena of macro-physics and biology that their introduction here would simply confuse our discussion.

We shall take the time to be that of the physics of Newton, or else of its main offshoot, that of Gibbs. The interesting thing about the time of classical Newtonian theory is that the laws which are the fundamental statements in which this time occurs read the same way forward and backward. This is no trivial statement. The one place in which Newton's laws have been triumphant with the minimum of extraneous assumptions is in the gravitational mechanics of the celestial bodies, and especially of the solar system. Their extreme success has been shown in the length of time for which we can predict the movements of the planets. It is possible to compute the nautical almanac for years in advance. Now, except for the minute effects of tidal friction and similar phenomena, the

methods of prediction which lead us to the nautical almanacs of the future are available with no modification whatever except that of the reversal of all velocities for the extension of the nautical almanac into the past. The music of the spheres is a palindrome.

This time of astronomy has been subjected, by Bergson, to a most severe criticism. The time in which we live has an obvious direction and cannot be reversed without the production of effects which are more than paradoxical. Two beings with oppositely directed times meeting one another would pass without any possible means of recognition. One would have his experience in his own past, which would be the future of the other, and they would share no common basis of experience. This time of living things is called by Bergson "duration" of life, thought, memory and meaning. Its one-directionalness is indicated by the fact that a purpose in the present can act only in the future. Simplify the purpose of a man into the tropism of a sperm and it is still true that the tropism of the present appears only as action in the future. In short, the time of Newton is appropriate for the consideration of a clockwork, and even among these, only of clockworks like the Leibnitzian monad, wound up in the infinite past, and not for the consideration of a sentient being whose future behavior is conditioned by past experience.

Planetary astronomy is the most ancient part of physics, and embodies what we try to achieve on earth by clockwork. Compared with the other natural sciences, it occupies an aberrant position. It is a domain in which experiment is impossible, but in which the ideal experimental position of the completely isolated system is more nearly fulfilled than in any other science. The astronomical system has only a small number of significant degrees of freedom, and the approximation which we make by assuming the planets either mass-points or ideal rigid bodies is surprisingly good. The forces we neglect in doing this, *i.e.*, the forces from outside the solar system, the forces of light and other non-gravitational activity, and the frictional tidal forces, are minute compared with those which we fully consider. No laboratory insulation of apparatus from external mechanical and electrical forces approaches the natural insulation of the solar system.

In the nineteenth century, the physics of the astronomer came to occupy a smaller angle of our mental vision, and the physics of the laboratory, the physics of the engineer, a progressively greater angle. Here, the causal isolation of an experiment is not something that nature offers to us without effort on our part, but something that we must seek by an elaborate ritual. The very notion of experiment involves the idea that natural situations are not isolated but can be repeated, at least in their essential aspects. From the beginning, this presumed something more than the mere spontaneous working-out of a single system with a single set of initial conditions, but it is only in the memory of scientists still alive that this presumption has received a clear analysis.

At the end of the nineteenth century, it seemed to many scientists

that the future of physics was to be one of continually seeking out the next decimal place. It was thought that physical sciences had reached their final form. Newtonian physics seemed adequate to all its problems—at least within the precision already achieved. In light and electricity, the work of Maxwell appeared about to give to his theory the permanence already attained by Newton's. Materialism had apparently put its own grammar in order, and this grammar was dominated by the concept of energy.

There were, it is true, already signs that the Newtonian frame of physics was being overstrained. Maxwell had made his great contributions to the kinetic theory of gases, and in this theory the initial state of a gas is conceived as a random aggregate of particles. However, the single aggregate in itself *cannot* be random. In any single aggregate, each particle is in a single definite position and the consideration of any unvarying condition of this sort does not contain the elements necessary for an adequate gas theory.

The new notion which has been added is that no system is random in itself, but can become random only by losing its identity in a set, or ensemble, of systems. More than that, an ensemble is not adequately defined by the mere listing of the individual systems it contains, but requires the notion of a distribution of these systems. This notion of distribution is equivalent to the notion of probability. Thus, the single Newtonian world evolving in a unique way from completely determined initial conditions is replaced by the notion of a family of Newtonian worlds, distributed according to some notion of probability, each of which revolves in a Newtonian manner.

This notion of the ensemble is implicit in Maxwell but becomes explicit, gradually, only in the work of Boltzman and of Gibbs. It is greatly extended in its scope. The Maxwellian notion of probability applies only to systems of a very large number of degrees of freedom, while Gibbs' will apply where the number of degrees of freedom is arbitrarily small. Furthermore, we owe to Gibbs the sharp notion of statistical equilibrium. In such an ensemble, the individual system may wander around in its confines but the probability that it will find itself in a region does not vary with the time. The class of regions involved is not sharply specified by Gibbs. We owe the precise interpretation to a man whose work in this field belongs almost exactly to the turn of the century, whose techniques and repertoire of ideas were completely foreign to those of Gibbs. This man was the French mathematician Henri Lebesgue.

The direct antecedents of the work of Maxwell, Gibbs, and Boltzman are to be found not so much in the Newtonian physics as in the classical thermodynamics. This represents a science in no way dependent on the Newtonian principles, but of a parallel development. It goes back to the eighteenth-century ideas on the nature of caloric, but is not dependent on that hypothesis and has a history of its own. Among its great names are Carnot and Joule. These writers occupied themselves with the in-

ternal economy of the thermal engine. Their great discovery was that heat flows from a source of higher temperature to a sink of lower temperature and that only in this flow can it be converted into useful mechanical energy. The fundamental discovery of thermodynamics was that, although heat is equivalent to mechanical energy and can be converted into it quantitatively, the only available source of mechanical energy is the cooling of a hot body and the consequent heating of a cool one. From this exclusion principle, it is possible to set up the entire system of formulae which has governed the development of the thermal engine.

This principle which we have just stated is known as the second law of thermodynamics. In the terms of the second law, it is possible to define a certain quantity which is always increasing, or at least non-decreasing, with time, and this we call the *entropy*. The very existence of an entropy is in contradiction to the Newtonian idea of time as applied to a single mechanical system. The great achievement of the three writers we have named, Maxwell, Gibbs, and Boltzman, is to have shown how, by the introduction of the notion of probability, the harmony between the Newtonian principles and those of thermodynamics could be reestablished. This reestablishment, though not in contradiction to Newtonian physics, was only rendered possible by bringing in ideas of a nature apart from those considered by Newton.

The direction of time is toward increasing entropy. Indeed, we can so define the direction of time. Another way of describing this direction is to say that, at any past time, observation and a certain *a priori* point of view have given us a probability distribution for events of that time, and a probability distribution for future events which represents the Newtonian working-out of the original one. Now, when we come to that future time, observations will have been made which were not originally in our possession. The combination of these observations with the probability belonging to the earlier time produces a new contingent probability which can be defined in terms of these observations and the original probability. The fact that this contingent probability almost always exists is another form of the mathematical theorem known as the fundamental theorem of the infinitesimal calculus.

The new data which we introduce between the time of our first observation of a system and the time of our later inspection may be called the experimental conditions. To perform an experiment is to submit an ensemble with an already determined probability to specific conditions which modify its later probability. This is an act which is humanly possible, and its counterpart in the opposite direction of time is impossible. To perform an experiment, we must take the system, modify its conditions, and then uncouple it as well as we can from outside influence for a stated time. To perform the experiment in reverse, we should have to have a system uncoupled until a given time on which we then perform a stated action. It is not, however, in our power to find a system which has been so uncoupled for all eternity. The fact of our intervention in select-

ing the system is in itself a coupling; thus, the equivalent of an experiment with time reversed is something we are not able to perform. In other words, the statement of the direction of the flow of time is not only a statement concerning the external phenomena with which we are dealing, but also one concerning our own directedness.

It is, of course, possible to ask whether in a science like astronomy, which is not one of direct human intervention, we have the same directedness of the time stream. It is even conceivable that the direction of the astronomical time stream, as measured by the direction of the increase of entropy, might be different in different parts of the universe. Perhaps we should regard the statement that entropy as observed in astronomy seems to be increasing in the regions of the world to which we belong as a statement of fact rather than of philosophical necessity. However, except for the very limited region of the solar system, we really have much less evidence than we often suppose we have that the direction of time has been read correctly by the astronomers.

One of the surprising things about Gibbs' work is that, though his main ideas have stood the test of nearly fifty years, his formulation of them is in almost every case wrong or even absurd. He considers the family of systems with the same energy. This is, in general, a set of several dimensions. He supposes that, in the course of time, each system flows through all the points of this set. On this basis, he tries to prove that the distribution of these points in space is the same as the average distribution in time of the points through which the individual system flows.

Now, it is not true that a dynamical system can actually run through every position admissible in its ensemble, but it is possible that its course be such that for every one of an extensive class of subsets of the ensemble, known as the *measurable* subsets, the fraction of time that the system stays within the subset may approach a definite limit as the time observed becomes longer and longer. Indeed, it has been proved by Birkhoff that in a dynamics in which the probability of any measurable ensemble can be considered finite and in which the probability of any measurable subensemble does not change with the flow of time, all systems spend a definite average proportion of the time within any given measurable subensemble, except for a set of systems of probability zero. If, in addition, every measurable subensemble that is invariant under the flow of time either has measured zero or that of the total ensemble, it has been further proved that the fraction of time which a dynamical system spends in a given subensemble is proportional to the measure of that subensemble. Such systems are called *metrically transitive*, or simply *ergodic*, and the properties of ergodic systems are amply sufficient to establish everything that Gibbs really needed for his new physics.

As stated by Gibbs, the new physics, although it contained a notion, probability, absent in Newton, was completely Newtonian as far as the individual constituent system was concerned. It is interesting to note that the development of physics in the present century has retained the



notion of probability while radically remodelling the Newtonian laws. In the quantum physics of Heisenberg and Schroedinger, we still have an ensemble of individual systems, but these individual systems no longer develop in a deterministic way. The systems of quantum statistics may, indeed, coincide in past history and then begin to diverge in a manner whose distribution is perfectly determinate but whose individual courses are unpredictable.

We now propose to introduce ideas belonging to the Gibbsian statistical mechanics into the theory of communication. This theory covers what is classically known as communication engineering and a number of other fields as well. The theory of the telephone is, of course, communication engineering, but the theory of the computing machine belongs equally to that domain. Likewise, the theory of the control mechanism involves communication to an effector machine and often from it, although the machine may not be watched by any human agent. The neuromuscular mechanism of an animal or of man is certainly a communication instrument, as are the sense organs which receive external impulses. Fundamentally, the social sciences are the study of the means of communication between man and man or, more generally, in a community of any sort of being.

The unifying idea of these diverse disciplines is the *message*, and not any special apparatus acting on messages. In particular, communication engineering is not in any essential way a branch of electrical engineering. Indeed, the engineers have realized for a long time that the same techniques used in electrical engineering are also applicable in acoustics, and the modern phonograph is the result of an explicit collaboration between these disciplines, based on the identity of their principles.

The message, to convey information, must represent a choice from among possible messages. If all I can do is to create, at the receiving end of a communication system, an enduring state completely characterized in terms of its own past, then I cease to convey information. If I send one of those elaborate Christmas or birthday messages favored by our telegraph companies, containing a large amount of sentimental verbiage coded in terms of a number from one to one hundred, then the amount of information which I am sending is to be measured by the choice among the hundred alternatives, and has nothing to do with the length of the transcribed "message."

It is, perhaps, not quite as obvious that the message not only presumes an ensemble of messages to carry a meaning, but it presumes an *a priori* distribution over the ensemble. Thus, if in nine cases out of ten I send a dot each second, but in the tenth case omit it, it is only when I omit it that I am furnishing significant information. This method of transmitting information is familiar in the case of time series.

Thus, we see that the notion of message involves the two fundamental ideas of statistical mechanics: the ensemble, and the probability distri-

bution over the ensemble. One perhaps needs a little more explanation because the notion involves *entropy* as well. Entropy here appears as the negative of the amount of information contained in the message. It may not be obvious, at first sight, why the notion of logarithm occurs in the measurement of the amount of information. Let me point out that information from two independent sources accumulates additively while their probabilities are multiplied, and that a variable which increases additively while another increases multiplicatively is, except for a constant factor, the logarithm of the second.

Perhaps we may exemplify this in another way. Let  $A$  be a quantity to be measured, lying on the range between zero and  $B$ . Let  $C$  be the error within which  $A$  can be measured. Let us write both  $A$  and  $C$  on the binary scale. Then the number of digits in  $A$  will be the logarithm of  $A$  to the base 2, and the number of digits in  $C$  will be the logarithm of  $C$  to the base 2. The difference between these two numbers represents the number of digits we know about. Each digit has two possible values and only two, namely, zero and 1. It follows that the number of significant decisions between two alternatives which we make in our measurement is the logarithm to the base 2 of the ratio  $A$  divided by  $C$ . If, however, we consider  $A$  as the size of our universe and  $C$  as the size of a certain zone of confusion, the number of decisions will be the logarithm of the probability that a measurement lies in a certain zone of confusion, when the probability of the entire universe is 1. This probability is taken to the base 2, but a change of base in our probability system only changes the measure of the amount of information by a constant factor. Thus, essentially, amount of information is the negative of entropy.

In fact, it is not surprising that entropy and information are negatives of one another. Information measures order and entropy measures disorder. It is indeed possible to conceive all order in terms of message.

We have already seen that the notion of entropy in classical thermodynamics is associated with the second law of thermodynamics, and this generally appears as an exclusion principle in determining the constructibility of thermal engines. We may naturally ask, Does any similar exclusion principle appear in the theory of communication? The answer is yes. No communication mechanism, whether electrical or not, can call on the future to influence the past, and any contrivance which requires that, at some stage, we should controvert this rule, is simply unconstructible. Not only is this principle used in a way similar to the second law of thermodynamics, but it is really identical with it. Another completely equivalent statement says that, once a message has been formed, a subsequent operation on it may deprive it of some of its information, but can never augment it.

A very important sort of ensemble of messages is one in which (a) the message is conceived as extending in time from the infinite past to the infinite future, and (b) the distribution of messages is invariant under a shift of the zero of time. Ensembles of this sort represent a good approxi-

mation to what we actually find, say, in a telephone system. The statistical distribution of the messages on which we operate does not vary from second to second, and the messages are long in comparison with the intervals of time in which we inspect them. A family of messages with such invariance is said to be a *time series in statistical equilibrium*. In such a system, this shift of the zero of time generates a group of transformations preserving the distribution functions unaltered, and we may apply Birkhoff's ergodic theorem. If, in addition, we assume metric transitivity, we can use the Gibbsian equivalence between time averages and averages over the ensemble.

Communication engineering is the art of introducing specific transformations into messages and transmitting them from point to point. An example of an operation which we may wish to perform on a message is its prediction. That is, we may wish to shift it forward in time. This is an operation which cannot be performed with perfect accuracy, since it involves a violation of the second law of thermodynamics. Without further information, we can do nothing. However, it is a perfectly valid question to ask how we may shift a family of messages of given statistical structure forward in time with minimum error. A possible measure for this error is the average mean squared difference between the shifted message and the message we obtained by our apparatus. This is a well-defined quantity which we can minimize by the principles of the calculus of variations.

This problem may be treated in several different forms. On the one hand, we may try to solve the problem absolutely for all possible operations whatsoever. To do this involves a full knowledge not merely of some but of all the statistical properties of the family of messages. On the other hand, we may restrict the operators to a particular class. The most important class of this sort is the class of linear operators, in which an additive combination of inputs yields a similar additive combination of outputs. These are the operators realized by the classical electrical use of resistances, capacities, and inductances. If we restrict ourselves to such means, the only significant statistical aspects of the message are those embodied in what is known as the spectrum. The theory of prediction for this case is already highly developed and is used in the practical design of apparatus. The calculus of variations, here, furnishes an integral equation as a case of what is called the equation of Euler and Lagrange. This equation is linear, and belongs to the particular type discussed by E. Hopf and the author.

The idea of reducing design to a principle of minimization, although old in other branches of engineering, is new in the theory of communication. The reason is that the minimization can only be carried out from a statistical characterization of the family of messages, and this statistical Gibbsian point of view has heretofore been wanting in the theory of communication. It has been treated as a branch of alternating current engineering, where it has been relegated to that part of the theory which deals with steady states. Now, a message in a steady state is a contradic-

tion in terms. If a message can never diverge from the path it has been following into another, it is conveying no information and should be stopped.\* However, the starting and stopping of messages produces a new type of phenomenon, the transient. In a general way, the difficulties produced by an imperfect treatment of steady conditions and by an imperfect treatment of transients lie in opposite directions. The apparatus which will deal in a superlative manner with the steady-state input is extremely delicate and sensitive, and will oscillate in the wildest way when subjected to considerable transients. It thus appears that we must seek the proper balance between good steady-state behavior and good transient behavior. Just where this balance may be found depends on the probable frequency of transients in the message. This, however, is a statistical quantity.

Thus, it is an insufficient appreciation of the problems of transients which has led to the neglect of the methods of the calculus of variations in communication engineering. This neglect was largely due to the fact that the part of communication engineering with the best-developed practice has heretofore been that of the telephone. The telephone terminates in the human ear, a very accommodating instrument. In particular, the ear is surprisingly insensitive to the phase of a message; it is, indeed, very difficult to give a mathematical formulation of optimum performance when the ear is the receptor. The case is far different with television, and the defects of the existing theory made themselves felt even before the beginning of World War II. During the war, the technique of television was extended to radar, and here, too, improved theories were necessary. It seems quite certain that design by minimization of average error is the technique of the future.

Prediction is not the only place where this technique is appropriate, nor even the most important one. It always comes into play where we desire the best solution of a problem whose perfect solution would violate the second law of thermodynamics. Of these places, perhaps the most important is the design of the wave filter. Here, we have a message which has somehow become scrambled with another, unwanted message which we call a noise. The problem of unscrambling these and restoring the original message with as little alteration as possible, except perhaps for a lag in time, is the problem of filtering. It is used in radio to eliminate static and tube noise, and in the telephone system to enable us to channel a number of conversations on the same line.

The apparatus employed for this purpose has an interesting history of almost fortuitous inventions. It originated as a side issue in the construction of the Heaviside distortionless long line. Since then, development has been largely conditioned by a series of lucky guesses and improvements attained by hit-or-miss. Theories which describe the best filter for a specific purpose are non-existent.

\* Lao-Tse says "Existence renders actual—nonexistence useful" and "It is useless to be able to do what one cannot stop doing." (The author is indebted to Dr. W. S. McCulloch for these quotations.)

A wave filter separates two messages, of which we wish to retain one and to suppress the other. These two messages, called, respectively, "message" in the ordinary sense, and "noise," have certain statistical attributes. They are often completely independent, but not always so. We receive the messages combined, additively or otherwise, into one. We want to put this through an operation, which we may embody in an instrument, to restore it as nearly as possible to the true message, displaced forward or backward in time by a fixed interval. The latter is commoner and more important. We want to minimize the mean squared error between the retarded pure message and the received mixed message, and we seek to do this by an instrument whose characteristic, or complex impedance as a function of frequency, we are to find. This leads to a problem of minimization quite like the one for prediction. To make this notion more concrete, let us see something of the stages that are needed in the work.

The first stage determines irreducible error, *i.e.*, the error which cannot be reduced by any delay whatever. (Parenthetically, let us remark that delay puts at our disposal a larger amount of information concerning the mixed message and so may reduce, but never increase, the mean squared error.) Next, when we know the irreducible error, we can determine a reasonable delay, such that the delay error is not large in comparison with the irreducible error, the sense of "large" depending on the problem. It would, ordinarily, be useless to reduce the lag-error below a tenth of the irreducible error.

Once the lag is determined, we obtain a characteristic which is realizable in the sense that it gives us an operator on only the past mixture of the noise and message. It does not follow that this operator can be realized by a resistance-capacity-inductance network of a finite number of meshes. However, we have at our disposal an adequate theory of approximation to such operators by finite networks. In this manner, we finally complete the specifications for our wave filter. These specifications give us an optimum filter to fit the situation exactly, whereas the earlier methods designed filters to certain specifications concerning pass-bands, sharpness of cut-offs, etc., which stood in no obvious relation to the actual demands of a problem and had to be adjusted to these by the process of trial and error.

Another problem which has no perfect solution is the design of compensators. We very often have to use special pieces of apparatus, as, for example, when we convert electrical messages into mechanical messages, having characteristics which involve a delay but are very far from a pure lag. We can much improve such apparatus by a process called *negative feedback*. In this process, we take from the output of the machine a certain amount of information and combine it with the input so as to decrease the difference between input and output. In the example, this makes relation between the all-over input and the mechanical output less sensitive to the particular characteristic of the electro-mechanical

stage. But this improvement is not won without a risk. When the apparatus to be regulated involves too much delay, there are limits to the amount of feedback which can be used without bringing the apparatus into spontaneous oscillation. For this reason, it is often desirable to have another sort of compensating apparatus to be used either in the feedback loop itself or, perhaps preferably, in advance of it. This apparatus is to have that characteristic which, in series with the motor organ or the whole feedback loop, as the case may be, makes the overall characteristic as close to unity as the ensemble of messages permits. This implies that the mean squared difference between the input and the output of the compensated mechanism shall be as small as possible. It will not be zero. The statistical problem we then face is a more general form of the problem of prediction, where the operator to be compensated was a pure delay.

Besides the filter and the compensator, there is similar apparatus of a mixed character, used where the message to be reproduced has been combined with a noise before it goes through the series of compensators and mechanical transformers. The theory of its design is more complicated than that of the simple filter, but involves considerations of quite the same sort.

We wish to apply notions from the field of communication to the study of the behavior of living organisms and their nervous systems. To do this is to treat the nervous system like an automaton. The interest in automata is nothing new and was especially strong in the late 17th and early 18th centuries. Leibnitz, for example, conceived his monads as something very much like automata. These automata were governed by the principle of pre-established harmony and had no causal contact with one another during operation. They had been wound up by the Lord at creation and continued to keep time with one another like separate clocks, so that they appeared to communicate with one another; but this appearance is merely a deceptive consequence of their synchrony.

It is clear that these automata were considered to be clocks. As a matter of fact, the automata made in the 17th and 18th centuries were run by clockwork. The dancing figures of the great Strassburg clock or on top of a music box are examples in point. Clockwork, likewise, drives the orrery that imitates the motion of the solar system and, like the solar system, represents a causal structure insulated as far as possible from the rest of the world.

Once the spring is wound, the figures on a music box are not affected by anything else in the world. They are blind and deaf and perform their predestined dance. They are as different from living organisms as anything can be, for the most important characteristic of a living organism is its awareness of the outer world. This means that it is furnished with organs of coupling to pick up messages from the outer world that condition its future conduct. To consider this in the light of thermodynamics

and statistical mechanics is instructive. We have a system of high energy coupled to a message low in energy, but extremely high in amount of information, *i.e.*, of great negative entropy. This is unlike the usual situation in thermodynamics, where all the coupled systems enjoy high entropy. But it may happen in the development of such a system that the internal coupling causes the information, or negative entropy, to pass from the part at low energy to the part at high energy, so as to organize a system of vastly greater energy than that of the present instantaneous input.

There is no reason why sense organs should occur only in organisms, and, in fact, they do not. The proximity-fuse, which explodes a shell when the radar waves sent out by it are reflected by the target plane, is a sense-organ in the strictest meaning of the word. Television apparatus, strain gauges, apparatus for observing the ( $H^+$ ), which give us a sort of chemical tester, and microphones are other cases in point. The modern automaton makes full use of such means of coupling with the external world, quite comparable with that of the living organism. It is not blind and it is not deaf.

A living organism receives messages in order to use them. In all but the simplest organism, there are special organs designed (if we may use teleological language) for this. These organs, collectively, form the nervous system and the effectors. The closest mechanical analogue to the nervous system is the computing machine. These machines, which may well be incorporated into machines for controlling something, are of two types: the analogical computing machine and the arithmetical computing machine.

Let us take the analogical computer first. In its crudest form, it is seen in the towing basin and the wind tunnel. A scale model of a boat is towed through a tank or a scale model of a plane is buffeted by an artificial wind in a wind tunnel. The motions of the models are observed under standard conditions. The differential equations covering these motions undergo known changes with the scale of the model, and these can be compensated to a surprising degree by suitable changes in the velocities and forces concerned. In this way, data which would cost a fortune and a lifetime to get from full-scale apparatus can be obtained promptly and cheaply.

The next step beyond this use of models replaces the model by something which has a mathematical equivalence to the actual system, even though it does not look like it, such as the potentiometer used as a multiplier or the machines for solving partial differential equations made by Vannevar Bush. In the latter, the variables of a system of ordinary differential equations are represented by the revolution of shafts so connected by a network of multipliers, adders, integrators, etc. that the differential equation has a complete mechanical representation. The solution of the equation for given initial values is then worked out by the machine as it revolves.

All such devices have one fundamental drawback. Their accuracy is closely dependent upon workmanship, and no known workmanship makes it possible to give an accuracy of more than three or four decimal places. There are quite interesting mathematical reasons for this. An integrating device which appears as an essential component in most of them is a variable speed drive. The usual way of producing a variable speed drive is by friction. For this drive to maintain an accurate ratio, the regions of contact between the driving and the driven member must be small. If, then, they are to transmit forces adequate for the operation of the apparatus, the space densities of these forces and, therefore, the pressures must be high. However, high pressures tend to act destructively on all but the hardest materials, and even with these they have limitations. These difficulties can be reduced by correct design and the use of servo-apparatus, but they always remain in one form or another and limit the performance.

As an alternative to the analogical, we have the arithmetical computing machine. The unmechanized form of this is the abacus. It has a number of balls whose position along rods indicates the numbers. We perform arithmetical operations by moving these balls according to certain rules. The interesting thing is that it is not the precise position of the balls that is important but merely whether they lie well within certain specified regions along the rods. When the apparatus is mechanized and the operations that have been performed in the head of the user of the abacus are replaced by operations performed, say, by entrained carriers, it is still true that so long as the part is within a certain range of positions it conveys one meaning; and the sources of error to which it is subject are such that the probability that it is in the wrong range is enormously small. In other words, we have a system which is like a surface with a lot of holes in it with sharp ridges between each hole. If we throw a ball on to such a surface we need only to put it in the region sloping to a particular hole to have it get there. The chance that it will find itself just balanced on the sharp edge between two holes is negligible. We have replaced precise positions by regions of attraction and the equilibria belonging to these regions.

There has been a progressive tendency with such arithmetical or digital computing machines to run them in the scale of two instead of the scale of ten. In the scale of two, we may write 7 as  $4+2+1$ , *i.e.*, as  $2^2+2^1+2^0$ . One way of writing this will be to omit the powers of two and merely give the coefficients, thus, *III*. Five, for example, is *IOI*. In this notation, all arithmetical operations are carried out exactly as on the scale of 10, with the advantage that the whole multiplication table reduces to:

	0	1
0	0	0
1	0	1

On the binary scale, there are only two regions of equilibrium for each



digit and every number is represented by a series of choices between two alternatives. Diverse as the particular operations of such a machine may be, the general principle is obvious. We start with a certain set of choices at the next stage. These determine choices for the third stage and so on, until the machine has completed its work. It is quite as easy to work with numbers of 10 or 20 digits as with those of 3 or 4, so that for more precise work the arithmetic machine is preferable by far to the analogical one.

The arithmetical machine has recently been developed to an extraordinary extent to solve problems which demand longer computations than would otherwise be practical at all. Here, advantage is taken of the extreme rapidity with which the fundamental element, the vacuum tube, can slip from one state to another. Problems requiring this high-speed operation include, notably, the solution of partial differential equations. The chief trick in them is scanning, the devices for which have come very largely from television. In order to handle partial differential equations, it is necessary to be able to represent and to operate on functions of 2 or more variables. There is no continuous way in which we can run over all the values of these functions in a finite time, but we may arrange these values in a lattice of two or more dimensions and run over the points consecutively in a manner closely analogous to that used in television. Here, the number of values we have to deal with is so great that any attempt to handle them manually is a waste of time and labor. Thus, our computing machine must be designed for speed, above all things.

A computing machine designed for speed must handle its data at this speed in all stages. Any human intervention makes the actual speed that of the human intervener and not that of the machine. All rules for the treatment of the data must be built into the machine from the beginning and only the final results can be available for human inspection. This means that the machine must handle not merely numerical data but also the instructions concerning the order of operations. In other words, every high-speed computing machine is *ipso facto* a high-speed logical machine. The fact that the logic of propositions like the binary number system can be expressed in terms of series of choices and operations thereon makes this possible.

Before we go into the detailed anatomy of the computing machine, it is perhaps as well to bring out, at the very beginning, the relations to the living organism. In the first place, complexes of processes involving varying quantities are to be found in the body. One of the places where they are most conspicuous is in the hormonal system. Here, we have a number of secretions which control the secretion of others, resulting in a complex balance of their actions. This process resembles the analogical computing machine more than the digital. The digital machine, on the other hand, is very like the nervous system. It is now generally agreed that, normally, the neurons have only two states of activity, impulse and rest. It is also likely that the state of a given neuron is largely determined

by the simultaneous states of the incoming neurons connecting with it. If the proper combination of incoming neurons fires, the next neuron fires and otherwise not. It is, indeed, possible that the thresholds to stimulation are determined, in part, non-neurally, say by chemicals, but the characteristic behavior of nerves is thoroughly like the relays in the arithmetical computing machine.

Whether our computing machine be artificial or natural, if it is to operate with no intervention but what enters through sense organs, it must be able to store data and recall them when they are needed later. The storage needed is of two different kinds: one, for the immediate operation of the machine, must be written down rapidly and must be rapidly accessible to the machine, and, when necessary, rapidly erased. The other serves for data which we desire to keep for a long time, either as part of the final result of the machine or in order to condition its behavior much later. Here, permanence is more important than ready erasure. Among methods which have been suggested for the first sort of memory are circulating processes. A message can be put into a delayer which will erase the message but rewrite it after a certain time. There are certain dangers in this process. Usually, a message gets blurred in transmission; it does not take many consecutive blurs until it is unintelligible. To avoid this, the message should be copied, so that instead of transmitting a blurred image of itself it recreates a sharp image. Instruments to do this are well known in electrical engineering; one of them is the telegraph repeater. In a circuit with one of these, unless blurring is extreme, the message will repeat at a similar stage with no blurring at all for an indefinite period. Mistakes and blunders need not accumulate.

In the nervous system, also, there is evidence of circulating memories. Here, the process is activity in a circular chain of neurons which keeps up indefinitely, once started. The neuron itself serves the function of the telegraph repeater. To work in the most efficient way, a computing machine must have certain parts which are assigned rather general functions. Connectors and memory apparatus will be available to any part of the machine as telephone trunks in an automatic switching system are available to any subscriber by a process of search. The searching need not be a consecutive opening or closing of switches as in the usual automatic telephone central. It may take more space and less time, as happens in organisms. However the searching is carried out, it is advantageous to recode items which have often occurred together in past searching, so that the process of searching for one facilitates the process of searching for the other. This coding and facilitation, highly desirable in a computing machine which must carry out long chains of processes without intervention, includes association and, generally, the perception of *Gestalten* or shapes. It is important that we be able to perceive a square as a square, whether it is big or small, whether its edge or its corner is toward us, whether in a vertical or horizontal plane. This means

that all of these perceptions must be brought close together in the coding system. One way to do this mechanically for geometrical figures is by running through all remembered transformations of a square which we should still call squares and comparing them with the given squares. This is unquestionably not the way that it is done in the nervous system. We have much evidence of coding in the special senses, particularly vision, where the number of receptors in the eye is so greatly in excess of the number of fibers in the optic stalk that some sort of coding must take place. However, the discussion required by this must be very precise and detailed and we are not now ready to go into it.

Up to the present, we have been considering the brain as the analogue of the computing machine, without discussing very much what happens to the information furnished by such a machine, *i.e.*, its output. In the office machine, this information appears in the form of numerical tables or of curves. However, there is no reason why the results should not appear as turns of a shaft or the opening or closing of valves or in any way whatever. Thus, the computing machine furnishes us the greatest promise for an adequate central nervous system in future automatic-control machines.

An adequate control apparatus should not only determine what its effectors are going to do, but should make sure that they have done it. For example, in the locks of the Panama Canal there is a control room which contains not only master valves for the sluices, signals to the towing locomotives and so on, but telltales actuated by the water level and the position of the locomotives, so that the operator may be sure that his commands have been executed and will not base a future command on a misconception. Monitoring the process of control is quite as suitable to an automatic-control machine as to one controlled by men. Among the information we receive should be the actual performance of every part of the machine. Fortunately, we have thermostats, strain gauges and the like, as well as a theory of feedback, to enable us to carry out these requirements.

We have already said a word or two about feedback and its limitations. If we exceed these limits, we are immediately faced by instability and oscillation. It is an interesting question whether similar phenomena of feedback, instability, and oscillation are found in animals. Feedback is vital to all voluntary activity. When I pick up an object I never know what muscular contraction will effect my end. How do I regulate my motion and why does it succeed? In each stage, I regulate my motion by the amount by which my task is not yet accomplished. This makes it possible to accomplish the same task regardless of my initial position and the object to be picked up. I monitor the extent to which I have completed my task in part visually, but also, to a considerable extent, kinesthetically. There are, in each muscle, sensory end-organs like strain gauges which report to the nervous system the stretch or rate of stretch to which the muscle is subjected. These can fail, and when they do we

are faced with a neuromuscular disease which may be crippling, as paralysis, although the muscles still respond to the orders we give them. But we do not know whether they have so responded and do not know what to do next. The tabetic patient, with a degeneration of the sensory fibers in the spinal cord, cannot stand up with his eyes closed. He can walk only by peering ahead of his feet and throwing them forward, since he must judge their position by eye, in place of the tactile and internal end-organs. His ultimate condition is miserable in the extreme.

Not only the absence of feedback but also its malfunction may produce well-known diseases. The victim of cerebellar injury can start a voluntary act but overshoots his mark, and in swinging back overshoots it still more. He goes into a tremor which appears only when he tries to carry out a voluntary act. His behavior is what one would expect if the regulation of the feedback necessary for the motion were impaired.

MacColl, in his excellent book on feedback mechanisms, indicates problems of very moderate complexity in which a stable feedback regulation may be obtained by two feedback loops but not by one. If, for example, we try to steer a ship automatically by using the gyrocompass directly, so that the departure between the ship's course and the indicated course opens the throttle of the steering engine, throwing the rudder hard to port or hard to starboard, we shall find that it goes into spontaneous oscillation of a serious type. If, on the other hand, this feedback chain: steering engine→rudder→ship→gyrocompass→steering engine, is supplemented by another feedback stage in the steering engine itself, so that the orders from the gyrocompass affect the throttle of the steering engine also in such a way that the motion of the steering engine itself varies the rate at which the rudder head varies the rate at which the position of the rudder head turns the throttle down, it is possible to get good steering. The importance of this is that most of our motor processes are quite complicated and demand multiplicity of feedback. To move our hand, we must in some way determine the position of our body, our shoulder, our elbow, our wrist. Only one of these stages of feedback is voluntary and fully conscious, and the other necessary stages belong to our very complicated postural mechanism. This is so far from being voluntary that it operates with a certain degree of competence even in animals whose cerebral hemispheres have been removed.

There are many conditions in which postural feedback is injured. One of them is Parkinsonism or paralysis agitans. In this condition, familiar among elderly people or people who have been subject to an attack of encephalitis, there is a continuous tremor which tends to be suppressed rather than emphasized when the patient moves. It is known that the central location of the mechanism of this disorder lies in the brain stem and not in the cerebellum.

Dr. Arturo Rosenblueth and I have made some attempts to apply the very precise existing theory of feedback mechanisms to the study of clonus in the nervous system. We took a spinal cat under strychnine

and attached the distal end of the quadriceps extensor femoris to a mechanism keeping it at constant tension. Under certain conditions of load, we found what was well known—that if it were tapped it would go into a continuing oscillation. We found that, if we were to take as our fundamental quantity the rate of firing of the motor neurons, the motion could not be regarded as linear. We found, however, that if we were to replace this measure by the logarithm of the number of motor neurons firing at a time, the motion became much more nearly linear, so that with certain not unrealistic formalizations of the data, we were able to predict its frequency of oscillation within adequate limits. I do not want to go further into the detail of this work, because much yet remains to be done with it both experimentally and theoretically. However, it represents a field of investigation which we intend to pursue in considerably more detail and to extend to neuromuscular processes of a much more complicated nature.

The history of the modern computing machine goes back to Leibnitz and Pascal. Indeed, the general idea of a computing machine is nothing but a mechanization of Leibnitz' *calculus ratiocinator*. It is, therefore, not at all remarkable that the theory of the present computing machine has come to meet the later developments of the algebra of logic anticipated by Leibnitz. Turing has even suggested that the problem of decision, for any mathematical situation, can always be reduced to the construction of an appropriate computing machine. There is, however, one difference between the situation in which we find ourselves when confronted with the ordinary artificial machine and that when confronted with a human being. The artificial machine is generally presented to us with all the rules of its operation explicitly stated, whereas the machine of the human being is very greatly conditioned by its own past experiences and is known to us only as one of a class of machines rather than as a certain specific machine. It may, therefore, seem that logical theory leaves the human machine freer than the artificial machine, so that while the artificial machine has one fixed logic of its own, man may wander through all logics. I think this view is false. The human machine, in any particular case and in any particular situation, has a determined logic, but we do not know precisely what it is. Nevertheless, this indetermination may force us to treat it as if it had in its actual power all the logics that all human machines have or even all the logics in some still more extensive class.

One important fact about the computing machine as well as the brain is that it operates in time. The certain results of mathematical logic are those which can be obtained from a machine. When it has worked out an assigned problem, it has nothing more to do and shuts down. Now it is not necessary for a machine to have this kind of asymptotic behavior at all. It may also go into permanent oscillation and keep saying *yes, no, yes, no, yes, no*, forever, or at least till our patience gives out and we turn it off. It may also involve an infinite process of a more complicated

nature which, though not repetitive, has no final termination. It is quite possible that this is what a machine would do when set to solve one of the perhaps unsolvable problems of mathematics such as Fermat's last theorem. In neither case will the machine be able to cope with the *Entscheidungsproblem*. The first case is the Russellian paradox, and the second the finitely unsolvable problem. The practical use of computing machines must reject these possibilities and must be confined to problems which can be solved and to machines which finally clear themselves.

Here, again, there is an important distinction between the artificial machine and the nervous system. The artificial machine carries the special data and special numbers needed for a specific operation, say, on a tape. When the machine has done its task and settles down to rest, these data are removed and replaced by other elements or by others of the same kind, and the machine is set up for another problem. But the human machine is never completely cleared. It always retains memories, from the past, of every situation which has ever confronted it. The depth and permanence of these memories is indicated by the success of a hypnotist or psychoanalyst in summoning them up from the depths. In other words, we can regard human life only as one grand problem and its separation into particular smaller problems as relative and incomplete. This coupling of all problems to all previously undertaken problems greatly complicates the behavior of the brain and may significantly contribute to its pathology.

There is another way of dividing machines into two classes which is relevant to the nature of the human mind. In one sort of machine, every element, either because of the physical structure of the machine itself or because of its permanently fixed memory, has a singular and immutable function. If a piece of information is to be remembered or a certain connection is to be used, it must be by the mediation of physical elements which have been assigned from the beginning for that purpose. The other possibility is to have a pool of connectors and mnemonic elements so that when we remember something or connect diverse elements we search until we find free elements and then switch them on. This second method is followed in automatic telephone switching, where there is no assignment of fixed outside trunks to subscribers, but where the machine answers a call by searching step by step for a free trunk and then making the connection. It is probable that the human brain functions in some such way, although it is unlikely that the resemblance between the two ways of searching is at all close. In a system in which connections are not provided in advance but are found by search, there is a probability that the connections be not completed. In the telephone system, there is always a possibility of finding all trunks busy. Now, serious as this is there, it is more so in the nervous system, because nervous activity involves connections of more than two stages and simultaneous activity within only a small section of the connected stages. This depends on the nature of synaptic activity itself. Thus, the prob-

lem of "line-busy" is even more important in the brain than in the telephone system. Difficulties of this sort probably contribute greatly to neuropathology.

One interesting fact about these problems is that they become more acute with increasing size of the nervous system. Any efficient machine is like O. W. Holmes' "one-horse shay" in that its breakdown occurs simultaneously at all points. If it did not, it would pay to reduce material and organization in the region of the points which have not broken down when the final breakdown occurs, to the advantage of those which have. Now, if the probability of finding a free line is  $1-E$ , the probability of finding  $N$  consecutive free lines is  $(1-E)^N$ , and this decreases very rapidly as  $N$  increases. In particular, a hormonal change decreasing synaptic permeability even to a slight extent as far as each synapse is concerned may completely block all activity in a sufficiently large brain.

Now, there is a certain amount of evidence that the human brain has already reached the point at which this sort of block has become serious. Of all the mammals, man seems to be the one which has developed one-handedness to the greatest extent. This handedness is associated with the dominance of one hemisphere of the brain and a suppression of the control by the other. A relatively slight injury to the dominant hemisphere of a man will produce grave defects, whereas a much larger injury to the other hemisphere is a relatively trivial occurrence. Pasteur developed an injury to one cerebral hemisphere in a period of his career when he had still much great work to do. He suffered for the rest of his life from a rather mild hemiplegia, or one-sided paralysis. When he died and the right half of his brain was examined, it was found to have been almost completely destroyed. This degree of difference between the effects of injury to the hemispheres is far greater than that which befalls the lower animals. Furthermore, the effect of cutting the commissures connecting the two halves of the human forebrain is astonishingly slight. It seems as if the problem of working the human brain as a single unit were too complicated to solve and that we have had to transfer its higher functions to one half, reserving the other half for much less important uses. Why this should be the case is not too hard to understand. The human brain is one of the largest of all, and of the large brains it has by far the thickest cortex, relatively speaking. Now, if we double the size of a brain, keeping all gross tissues in proportion, we shall multiply the number of cells by 8 and the number of connecting fibers only by 4, since their length is also doubled. In other words, we have a relative deficiency of direct remote connectors between different parts of the brain, and if we have effective connections it is at the cost of a larger number of intermediate stages.

Thus, the crowding problem of the brain is of importance, very much as the traffic problem is of importance in every large city. Combined with the absence of a complete clearing process, we see that concomitant mental processes in the same brain are likely to compete for internuncial

space and memory space, and that this competition is likely to increase the traffic jam still more. It may be that, in the future, we shall be able to do something better with situations where circulating memories have led to bad traffic jams than to destroy a part of the connections of the brain by a frontal lobotomy or to intervene brutally in all synaptic connections by one or the other varieties of shock therapy.

Besides the long-distance connections of the brain, made by tracts of white matter, there are also the random connections of short fibers between near-by cells. The anatomical picture of the cortex suggests that we may usefully employ statistical methods in the study of its function. This work has been taken up brilliantly by Walter Pitts. He finds that, under many conditions, the approximation to the activity of the cortex should not be made on a basis of rest and the zero basal activity. Proceeding from this, he has developed the wave equations of the cortex. These promise to give an interpretation of the electrocorticogram. He has also suggested the exploration of the connections between the different parts of the cortex by suitable stimulation of regions and the observance of the frequency-response at several points.

Up to the present, we have associated a theory of communication with psychology and neurology of organisms singly. In connection with this, we have already seen that two or more communication systems, such as computing machines, may be coupled into a single, larger system. This is true whether the constituent machines are artificial or natural machines. The coupling of human beings into a larger communication system is the basis of social phenomena.

In evaluating society as a system of communication, we must distinguish between assertions which are significant to the member but of which the system as a system is not aware because of an inadequate method of communicating them to other members, and the communications which actually belong to the social stock in trade. It appears, for example, that one ant cannot distinguish the identity of another ant, but can distinguish whether or not this ant belongs to its own nest. This is done by smell, which may be regarded as a hormonal means of communication in the ant community. Therefore, the identity of an ant is not a socially effective piece of information, while in human society the identity of the human being is an effective piece of social information.

It can, thus, be seen that the degree of organization of society and the amount of information socially available may be either more or less than that available to any member. The existence of an efficient language and, in particular, the existence of a long-time store of written or oral tradition vastly increase the amount of communal information and the possible complexity of the commune. Nevertheless, it is interesting to see that quite a high degree of communal organization can be effected without, or with the minimum, use of speech, in this way: Emotion in one organism is known to be a potent excitant of emotions in others. With the lower animals, it is quite possible that smell is here a



more important factor than sight. It is even possible that the odoriferous substances produced by animals at times of sexual activity or by other emotions may have a direct hormonal effect when observed through the olfactory mucosa, although it is by no means necessary to make such an assumption. In any case, emotion is certainly contagious, and it seems reasonable that, when one animal in a group exhibits it, the consequent emotion in the others produces some kind of fixative that perpetuates the associations belonging to that instant in the brains of the others. The effect of this is that a single type of emotional signal or a very small repertory of emotional signals, by means of its association with contemporary experiences of others, may be used as a signal much more complex because all the other experiences at the time will be added to it. If I could communicate with another man only by photographs, and if I always carried a camera around with me so that I could choose the moments at which I snapped pictures, I could still convey a significant story to him by my sequence of choices. For example, if I wanted to convey the notion of yawning, I might take twenty consecutive pictures on occasions when somebody was yawning. Now, an ability to fix the moment in the brain of another when his emotions were aroused amounts to just this. The meaning of my communication does not depend on the symbols in which it is conveyed, but is furnished from outside by the actual experiences of the man to whom I speak. He will usually see a world related to the world of my own vision, though not quite the same, for there will be a difference in perspective. This difference of perspective will mean that what he perceives belongs to the same *Gestalt* as that of my own perception, but it may be something within a wide range of experiences belonging to that *Gestalt*. Thus, we see how this sort of emotional communication forces us to organize our experiences according to *Gestalten*, by the simple use of association. I have spoken of vision, but this process is not confined to that modality.

The subject of language is too complicated and carries us too far for more than a sketchy mention here. First of all, figures formed by non-linguistic communication are ideal material for communication by words, and indeed our process of learning words is nothing but a transfer to these figures of our communication of a specific acoustic or visual symbol to others. This shows in the actual process by which children learn, and perhaps most of all where the process of learning is harder and simpler, as in the deaf-blind. In the teaching of Laura Bridgman, the first step was to excite her emotion in the presence of an object which she could feel, and it was only when *rapport* had been established by the continual presentation of the same object that there was any hope of making the further step to a symbol. In the actual presence of the two, people and the object, this emotional non-verbal communication may be extremely rich. It has in itself not only the roots of intellectual communication, but equally well the roots of the passionate communication which we know as poetry and art. The chief value of language is not that

it enriches communication, though it certainly does so, but that it puts communication into a form which is transferable without the physical presence of the objects it concerns. This leads to writing, in which it is no longer necessary to confront the participants in communication.

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### *General Discussion*

DR. DAVID MCK. RIOCH (*Chestnut Lodge Sanitarium, Rockville, Maryland*):

It is an honor to be present at this Conference on Teleological Mechanisms, as this occasion seems to me to be of historical importance. Dr. Wiener and his co-workers have offered convincing evidence that the occult force called "purpose," which is implicit but hidden in the concept of "reflex reaction to a stimulus," may be rigorously dealt with by the operationally more precise formulation of "interaction in time." There are many other neurophysiological phenomena to which this method may be immediately applied, some of which have peculiar interest, such as the "scratch reflex" which is independent of the sensory nerves to the muscles involved. However, it seems more important to re-emphasize that the essential contribution is in the demonstration of a form of thinking. Dr. Wiener and Dr. Rosenbluth have not merely taken more factors into account, by a vague reference to "the whole." Rather the opposite, they have precisely formulated the data, showing that they are the *data of interaction*, and that by the use of a mathematical hypothesis it is possible to determine which data are relevant to an adequate description of a *reflex*.

Some two decades ago, Mead, in the field of social psychology, and a little later Sullivan, in the field of psychiatry, pointed out that the data of human relations are the data of interaction and that the concept of "the individual" is a hypothesis derived from such data interpreted in the light of axioms of unknown historical source in our culture. It seems to me that, at this conference, we have been taken a step further. It has been demonstrated how the concepts derived from one set of operations

on a "feedback" mechanism—*i.e.*, on interacting processes—may be tested by another set of operations on the same system, thus removing the interpretation of the significance of the data from personal opinion to consistent operational demonstration. We no longer deal with a hypothetical "reflex center" in neurophysiology, or with a hypothetical "individual" or "group" in the social sciences. Instead, we have a method for testing the relevance and accuracy of the data of interaction, or "feedback."

The validity of extending this method of thinking from the experimentally limited situation of the knee-jerk and clonus, in which the feedback is immediate, to those interpersonal (and social) situations which are limited by participant observation of the investigator, in which much of the "feedback" is predictive, receives support from the work of others on this program. I should like to call attention, however, to certain other implications of the phenomenon in which we are now participating.

We have speakers and audience seriously considering analogies between man and a machine, and neither deifying the machine nor depersonalizing man. This implies a marked degree of mutual respect—self-respect and respect for our fellows—and it further implies that we have lived our lives until now in a social order which emphasizes the importance of man to man—an importance probably never before attained, at least in recorded history.

That the fruits of such relative freedom from threats to our self-respect appear at a time when that security is being threatened, gives pause to think. It may well be that, as scientists and professional workers, we must sacrifice part of our coveted isolation in order to preserve what we want: security in free communication with our fellows.

# CIRCULAR CAUSAL SYSTEMS IN ECOLOGY

By G. EVELYN HUTCHINSON

*Osborn Zoological Laboratory, Yale University, New Haven, Connecticut,  
and The American Museum of Natural History, New York, N. Y.*

THE aspects of ecology to be considered regard primarily the study of the conditions under which *groups* of organisms exist. Such groups may be acted upon by their environment, and they may react upon it. If a set of properties in either system changes in such a way that the action of the first system on the second changes, this may cause changes in properties of the second system which alter the mode of action of the second system on the first. Circular causal paths can be established in this manner.

It is well known from mathematical theory, and is abundantly illustrated by other contributions to this publication, that circular paths often exist which tend to be self-correcting within certain limits, but which break down, producing violent oscillations, when some variable in the system transgresses limiting values. When a breakdown of the self-correcting system takes place in nature, it may be expected to end in disaster for some element in the system which consequently disappears. The original system is thus destroyed, to be replaced by another in which the lost element plays no part. It is, therefore, usual to find in natural circular systems various mechanisms acting to damp oscillations, and self-correcting mechanisms may be introduced at several points in the circular path. The importance of such a multiple corrective system has already been made clear, particularly in Professor Wiener's contribution. The resulting stability, which appears to characterize most ecological systems, while it permits their persistence, makes investigation difficult.

The systems to be described range from cases in which at least part of the self-regulatory mechanism depends on purely physical aspects of the structure of the earth, such as the disposition of oceans and continental masses, to cases where the self-regulatory mechanism depends on very elaborate behavior on the part of organisms or groups of organisms. No sharply defined categories can be recognized in this series. It is, however, convenient to establish a methodological division of the following kind.

When a circular causal system involving a group of organisms is described in terms of the transfer of some substance through the system, without employing any purely biological enumeration, such as the size of a population, the mode of approach will be characterized as *biogeochemical*. When a circular causal system is described in terms of the variation in numbers of biological units or individuals, or, in other words, in terms of the variation in the sizes of populations, the mode of ap-

proach is characterized as *biodemographic*. In general, the biogeochemical approach is appropriate to the simpler cases which would ordinarily not be considered as involving teleological mechanisms, and the biodemographic to more complex cases, some of which might be regarded as involving teleological mechanisms.

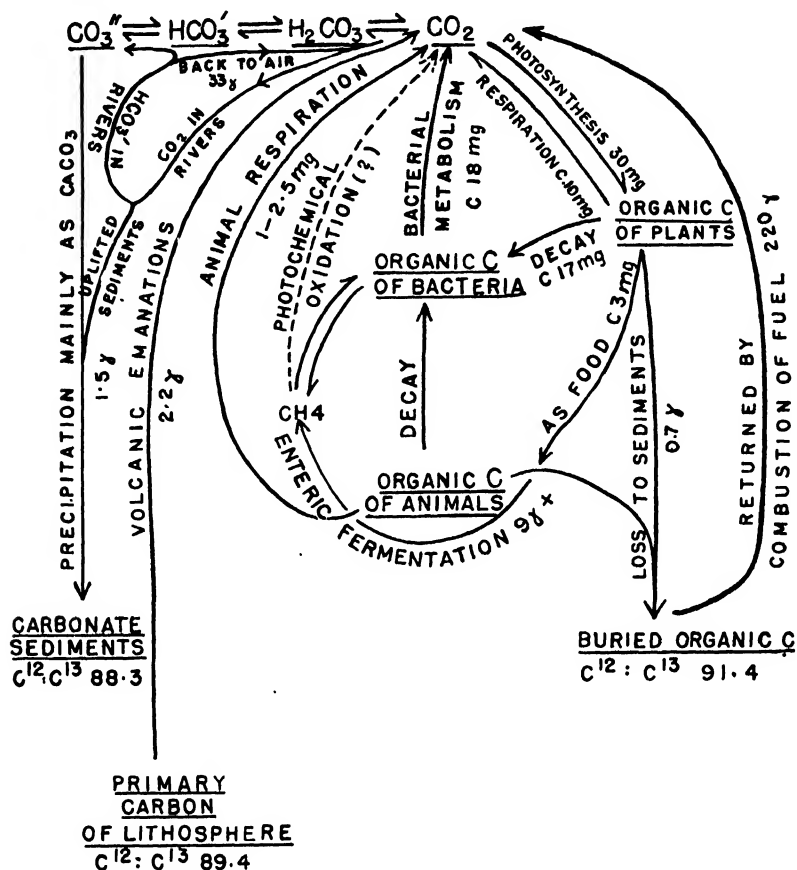
## THE BIOGEOCHEMICAL APPROACH

### *The Carbon Cycle in the Biosphere*

*The Biological Cycle.* In photosynthesis, green plants take up carbon dioxide from the atmosphere and hydrosphere and, using the energy of part of the solar spectrum, synthesize organic carbon compounds from the carbon dioxide. Part of the carbon dioxide is returned directly to the atmosphere in plant respiration. Most of the remaining organic carbon, produced photosynthetically, is returned indirectly either by the respiration of animals eating plants or by bacterial decomposition of dead plant or animal tissues. The qualitative aspect of this cycle is well known to every student of elementary biology. A quantitative statement is rarely attempted. The available quantitative information is summarized in the diagram of FIGURE 1, in which the rates of migration along the different paths are derived, from the works of Goldschmidt (1934), Noddack (1937), and of Riley (1944), supplemented by certain general considerations derived from the work of Lindeman (1941).

It has been pointed out by Kostitzin (1935) that a cycle in which the rate of growth of consuming and decomposing organisms depends on the rate of photosynthetic production, and the latter depends on the rate of return of  $\text{CO}_2$  to the atmosphere by decomposing and consuming organisms, would tend to oscillate according to Volterra's prey-predator equations. This oscillation would be accompanied by oscillations in the  $\text{CO}_2$  content of the atmosphere. Rough estimates of the period of oscillation can be made from the available data included in FIGURE 1. It is evident from such computation that the period would be of the order of magnitude of months or years rather than of centuries.

The problem of the constancy or variability of the carbon dioxide content of the atmosphere as a whole is a difficult one, owing to the large number of possible sources of purely local disturbance. The earlier data have been reviewed by Letts and Blake (1900) and, more recently, practically all the available information has been considered by Callendar (1940). It appears that the best series of nineteenth-century determinations, between 1866 and the end of the century, give mean values that vary very little, from 0.0287 to 0.0294 per cent. All series show variations dependent on meteorological and seasonal events, the highest mean content being in spring, 0.02971 per cent; the lowest, in summer, 0.02905 per cent. It is evident that any small variations due to inherent oscillations in the system would not be detected, but it is equally certain that

FIGURE 1. The carbon cycle (quantities as  $\text{cm}^2 \times \text{year}^{-1}$ ).

large oscillations, which might have effects on other systems, do not take place. During the twentieth century, there appears to have been a slight rise in the  $\text{CO}_2$  of the air, at least in the northern hemisphere. The nature of this is discussed below.

*The Regulatory Effect of the Oceans.* In considering changes in the  $\text{CO}_2$  content of the atmosphere, it is important to remember that the quantity of the gas in the air, equivalent to 0.11 gm. C per  $\text{cm}^2$  of earth's surface, is small compared to that in solution, mainly as carbonate and bicarbonate, in the ocean, equivalent to 5.5 gm. C per  $\text{cm}^2$ . Since the atmosphere and hydrosphere are in contact, the  $\text{CaCO}_3\text{--Ca(HCO}_3)_2\text{--CO}_2$  system of the ocean acts as an enormous shock-absorber regulating the quantity of  $\text{CO}_2$  in the air, as Schloesing (1880) pointed out long ago. This conclusion is usually accepted in the recent literature, though Callendar (1940) has supposed, on grounds that are critically examined be-

low, that the regulatory mechanism of the ocean operates so slowly that it cannot cope with the additional burden of  $\text{CO}_2$  produced by modern industry.

It is desirable to consider the path by which carbon dioxide enters the ocean. While some exchange across the surface must occur, and rain-water presumably carries a small quantity of the gas directly to the sea, the major contribution undoubtedly is derived from river water. According to Conway's (1942) analysis of the great body of data collected by Clarke, the rivers of the world discharge  $7.05 \times 10^{14}$  gm. of  $\text{CO}_2$  as carbonate, annually, into the ocean. While most of this carbonate is now derived from limestones, Clarke (1924) concluded that 8 per cent of the total quantity comes from the atmosphere. Actually, the atmospheric contribution to rivers, and so to the ocean, must be larger, because the data used are based on the residue after evaporation, *i.e.*, on carbonate rather than bicarbonate. It is reasonably certain that the mean river water will contain more than twice the amount of  $\text{CO}_2$  indicated by the carbonate content, for, not merely is it legitimate to assume that the bases are present as bicarbonate, but it also seems probable that there will be a slight excess of  $\text{CO}_2$ . Provisionally, therefore, it is probably safe to take 110 per cent of the observed value, or  $7.7 \times 10^{14}$  gm.  $\text{CO}_2$ , as representing the atmospheric contribution. At least the  $7 \times 10^{14}$  gm., or  $120\gamma$   $\text{CO}_2$  per  $\text{cm}^2$  of earth's surface present in excess of the carbonate  $\text{CO}_2$ , must be returned annually to the atmosphere, as the alkaline earth carbonates are converted into globigerina ooze and other carbonate sediments. The action of the bicarbonate  $\text{CO}_2$  of river water is therefore comparable to that of a conveyor belt, and the net direction of exchange across the surface of the ocean must be from the sea to the atmosphere. The available comparisons of the  $\text{CO}_2$  tension of surface water and of the air with which it is in contact (Krogh, 1904; Buch, 1939<sup>a</sup> & b) usually show a slight deficiency in the water, so that  $\text{CO}_2$  would tend to pass from the atmosphere into the ocean. The determinations have, however, all been made in the North Atlantic or Arctic during the summer months. As Buch points out, in regions of upwelling and seasons of much vertical mixing the situation must be reversed, for the deeper water of the ocean is known to be in equilibrium with much more  $\text{CO}_2$  than occurs in uncontaminated air. The overall excess of  $\text{CO}_2$  brought into the ocean, and liberated by carbonate sedimentation, must be returned to the atmosphere by such upwelling and vertical mixing.

*Gains and Losses to the Cycle.* Although the passage of carbon through the cyclical paths involving living matter and the ocean is the major event in the migration of the element, the cycle is not quite closed. Oxidized carbon is continually being lost to the lithosphere as carbonate sediments, and reduced carbon as dispersed organic carbon in shales, as well as in coal and oil, though these concentrates are quantitatively insignificant in the present context. The total quantity of fossil carbon in

the sediments is difficult to determine. For the carbonate sediments, Goldschmidt (1934) gives 6600 gm.  $\text{CO}_2$  per  $\text{cm}^2$  or 1800 gm. C per  $\text{cm}^2$ ; Noddack (1937) gives 8000 gm.  $\text{CO}_2$  per  $\text{cm}^2$  or 2200 gm. C per  $\text{cm}^2$ . The higher of these values, which are at any rate fairly concordant, will be used for reasons that will appear below.

The mean concentration of organic or reduced carbon in the sediments is taken by Goldschmidt to be about 0.2 per cent. This figure seems to be based not only on the available analyses but on certain considerations relating to the oxygen balance of the biosphere. Noddack gives a much higher weighted mean, namely 0.76 per cent C. This is based on an estimate of 0.94 per cent C in shales, for which no analytical evidence is available. The best analyses for shales (Clarke, 1904) give a mean of 0.81 per cent C, and taking the shales as constituting 80 per cent of the sediments, the weighted mean is 0.66 per cent, which in a column of sediment of 170 kg. per  $\text{cm}^2$  corresponds to about 1100 gm. per  $\text{cm}^2$ . The following considerations (Kamen, 1946) indicate that such a value is of the correct order of magnitude.

Carbonate precipitated from aqueous solution in contact with atmospheric  $\text{CO}_2$  is slightly enriched in  $\text{C}^{13}$  over the carbon of plutonic rocks. The carbon that remains after carbonate has been precipitated is, in consequence, slightly impoverished in the heavy isotope.

In a series of determinations of the isotopic ratio, Nier and Gulbransen (1939) found the mean value of  $\text{C}^{12}:\text{C}^{13}$  for primary carbon (diamond, graphite, meteoritic) to be 89.4. Three limestones of varying age gave 88.3, specimens of recent and fossil plant carbon 91.4. These results suggest that in the course of geological time about twice as much carbonate carbon as reduced carbon has been deposited in the sediments. A later study by Murphey and Nier (1941) confirms the difference between plant carbon and carbonate carbon, and indicates no difference between fossil and recent specimens. The results of the later study apparently cannot be used with the earlier investigation, because, although the differences between the ratios within each series are reasonably accurate, the absolute values are apparently subject to instrumental error. In the second series, the primary carbon samples are overweighted in favor of meteorites, only one terrestrial specimen being examined, so that it is difficult to know what value to use for the original material from which the carbon of plants and limestones came. Accepting provisionally the results of the first series, it is clear that they are in good accord with the geochemical estimate of 2200 gm. per  $\text{cm}^2$  carbonate carbon and 1100 gm. per  $\text{cm}^2$  organic carbon. Accepting a 2:1 ratio, if Goldschmidt's value of 1800 gm. of carbonate carbon per  $\text{cm}^2$  be more accurate than Noddack's, the corresponding reduced carbon would be 900 gm. per  $\text{cm}^2$ , corresponding to 0.53 per cent C in the mean sediment. The reason for Goldschmidt's conclusion that the reduced carbon content of the sediments is considerably lower than even this estimate, appears to lie in his consideration of the oxygen content of the sediments. During the



process of erosion and sedimentation, oxidation of the sediments takes place. The oxygen lost in such a process may be assumed to be of photosynthetic origin. An equivalent quantity of reduced carbon should therefore be fossilized. Goldschmidt finds that the maximum possible amount of fossil oxygen corresponds to but 0.17 per cent reduced carbon in the sediments, and this figure has clearly influenced his estimate. Actually, it seems inevitable to accept the lack of balance implied by the excess of reduced carbon in the sediments which presumably can be explained (Poole, 1941; Conway, 1943; Cotton, 1944) by the oxidation of methane, carbon monoxide, hydrogen, and sulphur dioxide in volcano gases.

The 2200 gm. per  $\text{cm}^2$  of carbonate carbon and 1100 gm. per  $\text{cm}^2$  of organic carbon correspond to 12,100 gm. of  $\text{CO}_2$  per  $\text{cm}^2$  of the earth's surface. In other words, since the beginning of sedimentation, approximately twelve atmospheres of  $\text{CO}_2$  have been removed from the biosphere. The available evidence strongly suggests (Conway, 1943) that this immense quantity of  $\text{CO}_2$  did not exist all at one time in the atmosphere, but has been added and removed gradually. It has long been realized that volcano gases supply the required source of carbon dioxide. No direct measurements of the output of the gas from volcanos are as yet available. The only estimate for the rate of production of volcanic  $\text{CO}_2$  that can now be made is obtained by dividing the carbon lost to the sediments by the geological time-span of  $1.5 \times 10^9$  years. The result is  $8.0\gamma$   $\text{CO}_2$  or  $2.2\gamma$  C per  $\text{cm}^2$  per year. Goldschmidt used a rather lower figure ( $3-6\gamma$   $\text{CO}_2$  per  $\text{cm}^2$  per year) for reasons that are already apparent. It has been pointed out before that  $7.7 \times 10^{14}$  gm.  $\text{CO}_2$  or  $132\gamma$  per  $\text{cm}^2$  are apparently brought into the sea, annually, by rivers. Most of this  $\text{CO}_2$  is evidently returned to the air, but Clarke concluded that 8 per cent of the  $\text{CO}_2$  of carbonates is of atmospheric origin. These 8 per cent or  $10\gamma$  per  $\text{cm}^2$  per year will be lost to the cycle and should be of the same order of magnitude as the  $8.0\gamma$   $\text{CO}_2$  which are known to be fossilized as carbonate, or after reduction. In view of the uncertainties involved, the agreement is highly satisfactory.\*

*The Efficacy of Self-Regulatory Mechanisms.* Callendar (1940), considering the best data available since 1866, concludes that during the present century there has been an increase of the order of 10 per cent in the  $\text{CO}_2$  content of the atmosphere. This he attributes to the modern industrial combustion of fuel. He points out that if the increase in  $\text{CO}_2$  content observed is uniform throughout the atmosphere, it corresponds to an accession of  $2 \times 10^{10}$  tons. The total addition during the period 1900-1935 from fossil fuel is taken as equivalent to  $1.5 \times 10^{10}$  tons of  $\text{CO}_2$ . In view of the difficulties in obtaining good representative series

\* Since this paper went to press, a good deal of work has been published bearing on the isotopic ratio of carbon from various sources. Some modification of the quantities given in the diagram will ultimately be necessary, but as the subject is in a state of flux it would be premature to attempt detailed correction at the moment; the orders of magnitude involved are doubtless correctly indicated. It must, however, be pointed out that an age of  $3.3 \times 10^9$  years for the earth is certainly more acceptable than that given above. This age reduces the  $\text{CO}_2$  output by volcanos to about  $4\gamma$  per  $\text{cm}^2$  per year. The divergence from the  $10\gamma$  per  $\text{cm}^2$  per year lost to sediments can hardly be regarded as serious.

of data on  $\text{CO}_2$ , the agreement would appear to be satisfactory. Callendar supposes that the regulatory mechanism of the ocean operates so slowly than an increase in  $\text{CO}_2$  at the rate implied cannot be controlled and that the whole of the industrial  $\text{CO}_2$  output of the first third of the twentieth century has remained in the atmosphere. There are, however, two very grave objections to accepting this conclusion.

The rate of industrial production of  $\text{CO}_2$  used by Callendar is  $4.3 \times 10^8$  tons per year, or essentially the same as that implied in Goldschmidt's estimate of 800 per  $\text{cm}^2$  per year. The corresponding value in terms of carbon is  $1.2 \times 10^8$  tons. Riley's most probable estimate of the total rate of photosynthetic fixation, and consequently of liberation of  $\text{CO}_2$  by respiration and fermentation, is  $1460 \times 10^8$  tons, while for terrestrial areas Noddack's value is  $151 \times 10^8$  tons. The rate of industrial production is therefore of the order of 1 per cent of the rate of biological production on land and of 0.1 per cent of the rate for the whole earth. Assuming the validity of Callendar's conclusion that transport through the sea surface is not involved, it is apparent that for his view to be correct it is necessary also to suppose that the mechanism of the biosphere is such that a very accurate regulation occurred during the nineteenth century, but that during the twentieth an increase of the order of 1 per cent in the total production of  $\text{CO}_2$  was quantitatively rejected by the system. This is extremely improbable.

In addition to this theoretical difficulty, there exist observational data of great significance. Glöckauf (1944) has determined the  $\text{CO}_2$  content of twelve samples of air taken from altitudes of 4,000 to 10,000 meters over Great Britain. His results range from 0.024 to 0.030, the mean being  $0.025 \pm 0.001$  per cent, which is slightly lower than the mean of the lowest series of nineteenth-century determinations. Analyses of air at ground level by the same technique as was used in the analysis of the upper-air samples gave values of 0.031–0.035 per cent in accord with other modern determinations. If Callendar's hypothesis be adopted, it is necessary to reject any free interchange of air above 4,000 meters since late Victorian times, which is absurd.

The true interpretation of these results would appear to be a slight change in the distribution of stationary concentrations of  $\text{CO}_2$  passing through the system, rather than a static accumulation of the gas. Since the upper-air values are lower than those at ground level, and since there is no obvious way for  $\text{CO}_2$  to be lost in the upper troposphere, a full elucidation of the problem must await determinations of the origin of the air masses in question. There must be sinks as well as sources in the atmospheric circulation of the gas. If, as seems probable, the sinks are local areas of ocean surface, they have not yet been discovered on a scale commensurate with what is required. Meanwhile, it seems far more likely that the observed increment in the carbon dioxide of air at low levels in both Europe and eastern North America is due to changes in the biological mechanisms of the cycle rather than to an increase in industrial

output. It is quite probable that the net effect of the spread of the technological cultures of the North Atlantic basin has been to decrease the photosynthetic efficiency of the land surfaces of the earth. Though the most productive agricultural land, on which corn is cultivated, can have a photosynthetic efficiency greater than that of the best temperate forest, it is very unlikely that, in general, cleared farm land is as biologically productive as is virgin forest. Moreover, it is obvious that long-term readjustments, due to increasing photosynthesis with increasing  $\text{CO}_2$  pressure over a period of years, are less likely to be effective on agricultural land because the biological community is annually built up again from nothing, while gains of one year in tree growth are likely to be reflected as increased photosynthetic surface in the succeeding year. The hypothesis that deforestation, perhaps coupled with soil erosion and loss of nutrients to the sea, has changed the composition of the atmosphere over the land during this century at least demands serious consideration. Whatever the ultimate solution of this problem may be, Glückauf's results certainly indicate that the self-regulating mechanisms of the carbon cycle can cope with the present influx of carbon of fossil origin, even though changes in the pattern of steady-state concentrations may have occurred.

A further aspect of this problem must be briefly considered. If the conclusion that has just been reached is correct, it is apparent that the addition of  $\text{CO}_2$  at a rate corresponding to a hundred-fold increase in vulcanism is not sufficient to derange the main self-regulatory processes. There is, therefore, little likelihood that a moderate decrease or increase in vulcanism during the past has had any significant effect on the overall regulation of the  $\text{CO}_2$  content of the atmosphere. Moreover, if the modern increase is correctly ascribed to human interference with the biological mechanism of regulation, it is improbable that changes in vulcanism *per se* could have produced significant changes in the pattern of steady-state concentrations. It is, however, reasonable to suppose that changes in the emergence of the continents may have had some small effects by altering the area of the terrestrial plant cover, while greater effects may be reasonably attributed to the major evolutionary steps in the development of the land flora.

*A Possible Change in the Productivity of the Ocean.* The only hint of any change in the quantitative aspects of the carbon cycle that can be derived from the geological record suggests that the Palaeozoic sea was slightly more productive than the Mesozoic or Tertiary ocean. Clarke (1904) gives the mean carbon content of fifty-one Palaeozoic shales as 0.88 per cent, while that of 27 Mesozoic and Cenozoic shales was found to be 0.69 per cent. The significance of such a difference is increased by the finding of Miller (1903; Hall and Miller, 1908) of a mean content of 0.65 per cent C in a collection of shales and clays, mainly Mesozoic, from Britain. If subsequent analyses confirm the slightly greater carbon

content in the older shales, it will be apparent that more organic carbon per gram of argillaceous matter was deposited in the Palaeozoic than at a later date.

*The Methane Cycle.* An alternative path, involving the liberation of methane to the atmosphere, is seldom considered. Though this process is much less impressive than that of the return of carbon to the air as  $\text{CO}_2$ , there clearly must be a mechanism for removal of methane, if it is not to accumulate. The data implying the existence of a minor but nevertheless significant methane cycle are derived from a consideration of the gaseous exchanges of large herbivores. Benedict and his co-workers have shown that fermentation in the alimentary canal of such animals gives rise to so much methane that a significant proportion of the carbon of the food ingested is lost in the expired air as  $\text{CH}_4$ . TABLE 1, which follows, has been prepared by Mrs. Jane K. Setlow, using the data for the world populations of domestic animals collected by Rew (1928) and those for production per day per animal given by Ritzman and Benedict (1938).

TABLE 1

	<i>Methane produced per day, gm.</i>	<i>Methane produced per year, kgm.</i>	<i>No. of animals, <math>\times 10^6</math></i>	<i>Total methane, <math>10^3</math> metric tons</i>
Horse	106	38.7	99.8	3,862
Cattle	200	730	510.9	37,296
Goat	14.7	5.4	116.8	631
Sheep	15.1	5.5	532.2	2,927
Elephant	470	171.5	0.5	86
TOTAL				44,802

The total production is probably considerably greater than the sum given in TABLE 1. Not only must an extensive but unknown population of large ungulates, notably antelopes, various species of wild Bovinae, and deer be considered, but also the large production of methane in swamps and lakes for which no satisfactory estimate is possible at present. It is probably quite safe to double the estimate given in the table, and not unlikely that the resulting figure of about ninety million metric tons is too low. Such a quantity corresponds to about 0.06 per cent of the annual photosynthetic fixation of carbon. It is obvious that unless mechanisms for the oxidation of methane exist, circulating carbon would slowly accumulate in the atmosphere. Three possible methods of oxidation appear to exist:

- (1) A small amount of direct combustion in lighting flasks and on hot lava surfaces may be expected.
- (2) Photochemical oxidation might be expected in the stratosphere.
- (3) Bacterial oxidation certainly occurs in soils.

The second and third mechanisms seem more likely to be important than the first, though both of them take place in regions more or less cut off

from the turbulent diffusion of the troposphere. It would, indeed, appear remarkable that the suggested mechanisms of oxidation are sufficient to keep the stationary concentration of methane in the atmosphere at an almost undetectably low level (Hutchinson, in press).\*

*General Properties of the Carbon Cycle.* It will be apparent that two main self-corrective systems exist in the carbon cycle, namely, the  $\text{CO}_2$ -bicarbonate-carbonate system involving air, sea, and sediments, and the biological photosynthetic cycle. The first of these involves a reversible chemical equilibrium, the rate of establishment of which is in part determined by physiographic factors, while the second involves an irreversible path which could theoretically give rise to oscillations but which probably does not do so because of the damping action of the first mechanism. The distribution of steady-state concentrations in the atmosphere probably depends on both mechanisms. It has undoubtedly altered in the past half-century, probably owing to changes in the vegetation cover of the earth. An alternative path in the carbon cycle, involving methane, certainly exists, though it is not likely to be of significance in determining the self-regulatory properties of the whole cycle.

Several other biogeochemical cycles involving passage through the atmosphere exist. By far the most important of these is the nitrogen cycle. In the cycles of sulfur and of the halogens, transport through the atmosphere also undoubtedly plays a part, but the quantitative details are little known. The nitrogen cycle is far more complicated than that of carbon. In the carbon cycle, the only important compounds circulating freely outside organisms are  $\text{CO}_2$  and carbonates among the oxidized carbon compounds, and methane among the reduced carbon compounds. In the nitrogen cycle, ammonia, molecular nitrogen, and several oxides are certainly present in the atmosphere, though the stationary concentrations of all but  $\text{N}_2$  are minute. Ammonia, nitrate, and often nitrite are present in the hydrosphere. Several alternative paths exist in the cycle. Cooper (1937) gives a good modern presentation. Quantitative biogeochemical data are, however, very inadequate. Most of the information that exists has been considered in an earlier paper (Hutchinson, 1944a). It is reasonably certain that both fixation of  $\text{N}_2$  and formation of  $\text{N}_2$  from nitrogen compounds are mainly mediated biologically. It is also obvious that the steady-state position relative to fixation and formation of molecular nitrogen is such that nearly all the nitrogen is in the molecular condition. There is, moreover, adequate evidence that the fixation process is highly inefficient and consumes a significant fraction, conceivably of the order of  $n \times 10$  per cent of the total energy fixed photosynthetically. The nitrogen cycle is thus linked to the carbon cycle. The linkage, moreover, involves circular paths, for not only is the total rate of entry of molecular nitrogen into biological systems likely

\* After the meeting at which this paper was given, Dr. F. H. Pike told the author that he had been informed that in Hungary, during the process of industrial separation of krypton and xenon from the atmosphere, explosions had occurred which were traced to a gas with about the boiling point of krypton,  $-151.8^\circ \text{C}$ . (methane boils at  $-161.4^\circ \text{C}$ .), and the concentration in the atmosphere of xenon.

to depend on the rate of photosynthesis, but the rate of photosynthesis is, clearly, in part determined by the steady-state concentration of nitrogen compounds available for the maintenance of plant populations. It is evident, too, that both cycles in part depend on the rate of liberation of phosphorus from the lithosphere on land and the rate of regeneration of phosphorus by vertical mixing in the sea. Thus, it is possible that the biological cycle of the elements must be regarded as a unity, the rate of working of which is primarily determined by non-biological factors, such as precipitation, winds, currents, and the disposition of the continents and oceans.

### *The Phosphorus Cycle in Inland Lakes*

*Biological Productivity and Phosphorus Concentration.* The second biogeochemical example to be discussed relates to much smaller systems than the entire biosphere. Such systems, being much younger than the biosphere, throw light on the mechanisms by which a steady state in the cycle is achieved.

In a series of small inland lakes of similar area and depth, there is evidence that the variation in the total quantity of living matter inhabiting the water and the mud depends primarily on the supply of phosphate and combined nitrogen that can pass from the drainage basin into the lake. All other nutrient elements are likely to be present in excess in inland waters. If a colorless bottle of lake water is suspended at the surface of the lake from a buoy, little change in the total quantity of phytoplankton in the bottle will occur in the course of a week. Addition of a small quantity of phosphate and nitrate, however, will in a similar span of time cause a great increase in the total quantity of plant material in the water enclosed in the bottle. Since no appreciable amounts of phosphorus enter the atmosphere, and since the phosphorus cycle rarely involves any compounds less oxidized than phosphate, this cycle presents simpler features than does that of nitrogen. The phosphorus cycle will therefore be used to provide the main basis for the argument, though in general a parallel movement of nitrogen compounds can be assumed.

At least during the summer months, the planktonic algae keep the concentration of phosphate at a hardly traceable low level, rarely in excess of a few micrograms per liter. The algal cells, most of which probably require 10 $\gamma$  per liter phosphate phosphorus for optimal growth, are evidently living in a state of chronic starvation, and any small addition to the nutrient salts of the water is presumably taken up rapidly. At the same time, algal cells are being eaten by animals; some part of the algal material is constantly being incorporated into fecal pellets, and these, being larger than the algae, sediment more rapidly.\* This rain of feces must constitute a drain on the phosphorus supply of the illuminated

\* There is evidence that many algal cells pass through zooplanktonic organisms undigested and so are sedimented intact.

upper water of the lake. Moreover, changing conditions of temperature and perhaps of illumination lead, directly or indirectly, to changes in the specific composition of the microscopic algal flora, so that populations of algae are continually dying and sinking. Animal deaths again must remove material in the same way. There is, thus, a continual falling of material from the illuminated water to the bottom of the lake, and this falling must remove the various constituents of living matter, among which phosphorus is notable. In shallow lakes, regeneration of the phosphorus can occur through the wind-generated circulation of the water, which will bring material that is diffusing from the mud into the illuminated layers where phytoplankton can grow. In deep lakes, the illuminated trophogenic waters tend to be cut off from the lower layers, which remain cold throughout the summer. A great deal of indirect evidence (Hutchinson, 1941) shows that enough horizontal movement of water occurs at all depths, even in very stably stratified lakes, to bring phosphorus from the mud into the open water. In a lake in which practically no vertical mixing of water is occurring except in the top meter or two, the phytoplankton is continually growing and sedimenting, thus removing phosphorus from the illuminated zone, and this phosphorus is continually being replaced, by movement of phosphate liberated by decomposition, from the mud into the open water. It has recently been possible to confirm the existence of this cycle by the use of radiophosphorus (Hutchinson and Bowen, 1947, in press).\*

The factors controlling the rates of the various parts of this cyclical migration are practically unanalyzed and are certainly very complex. Physical events external to the cycle possibly play a preponderant role and may prevent the recognition of any clear self-regulatory mechanisms when the process is studied over a short time-span. An indirect approach to the study of the problem over long periods of time is, however, possible.

*The Growth of the System.* The nutrient cycle in a lake is not quite closed. Small quantities of nutrient elements are being brought into the lake by the inlets and by the seepage of ground water. Small amounts are, likewise, lost through the outlets and to the sediments deposited on the lake bottom. At least in biologically productive lakes, the rates of such gains and losses are very small, compared to the rate of transfer through the cycle. Their existence, however, makes further analysis possible. When a steady state is achieved, the losses will just balance the gains, and part of the losses are available to analysis through the study of sediment profiles collected with various boring devices.

\* As described above, the cycle would deplete the mud in contact with the upper layers and enrich the mud in contact with the lower layers of water. This process would finally bring the cycle to an end. It is not certain why this does not happen, but it seems likely that the rooted vegetation of shallow water is involved. Such vegetation presumably stores much phosphorus derived from freely circulating water in the spring. Some of this phosphorus will have come from mud in the deep water, but it will all be returned, when the weeds decay, to mud in the shallows. Uptake of radiophosphorus from the free water by rooted weeds has been demonstrated.

Since the above was written, Rodhe (1948) has shown that some planktonic algae require very much less phosphorus than is indicated here, but the general statement probably remains true for the association as a whole.

If a lake sediment be poor in organic matter, little regeneration of nutrient substances will take place from it. There will be little organic nitrogen present to produce ammonia or nitrate, and though some phosphorus will probably be present in the inorganic fraction, it will be in the form of insoluble minerals. A little of such phosphorus can go into solution and support algal growth, as Strøm has shown in the case of the inorganic silts of Norwegian mountain lakes, but the rate of liberation of phosphate from such a deposit is likely to be very slow. If, however, the sediment be rich in organic matter, there will be much nitrogen to pass into solution as the result of bacterial metabolism, the phosphorus of organic origin will usually be freely soluble, and the carbon dioxide present in the mud may facilitate the solution of the mineral phosphates.

In temperate regions, where decomposition on the lake bottom is slow, the condition for the presence of primarily inorganic sediments is that little organic material be produced in the lake, the condition for the existence of a richly organic sediment being the production of much organic matter in the lake. Some additions from the vegetation of the catchment basin may be expected in all cases, but it appears that such additions do not alter the character of the process to be described.

Starting with a barren glacial basin newly filled with water, the concentration of phosphate in solution in the drainage basin will depend on the general geochemistry of the region. At first, the nitrogen available will probably be derived solely from rain water, but nitrogen will tend to be fixed biologically, according to the availability of organic matter, the production of which will depend on the availability of phosphorus and other mineral nutrients. The water of the lake will gradually develop a phytoplankton population. The first sediments to be deposited will be almost entirely inorganic, but as soon as remains of organisms are included in the surface layer of these sediments, an internal cycle of the kind already described will be established. Phosphate will be more easily liberated from mineral particles, owing to the production of carbon dioxide by decomposition, and the phosphate of the decomposing organisms will be returned to the lake water. As productivity increases, the sediments will become more and more organic, and thus more and more able to return the nutrients rapidly to the cycle. This process continues until the geochemically determined nutrient potential of the silt and the water of the drainage basin is fully utilized. In a *eutrophic* region, rich in nutrients, the final result will be a fertile lake, while in an *oligotrophic* region, poor in nutrients, it will be a relatively sterile lake (FIGURE 2).

The general interpretation just given was derived from a study of the sedimentary cores collected by Dr. E. S. Deevey in Linsley Pond, a small, rather productive lake in North Branford, Connecticut (Hutchinson and Wollack, 1940). It was apparent from a study of these sediments that initially the productivity of the lake was very low, but that it then rose with increasing rapidity. Later, the rise in productivity was checked,



and a long period set in during which some irregular variations, but no great divergence from an essentially constant steady-state condition

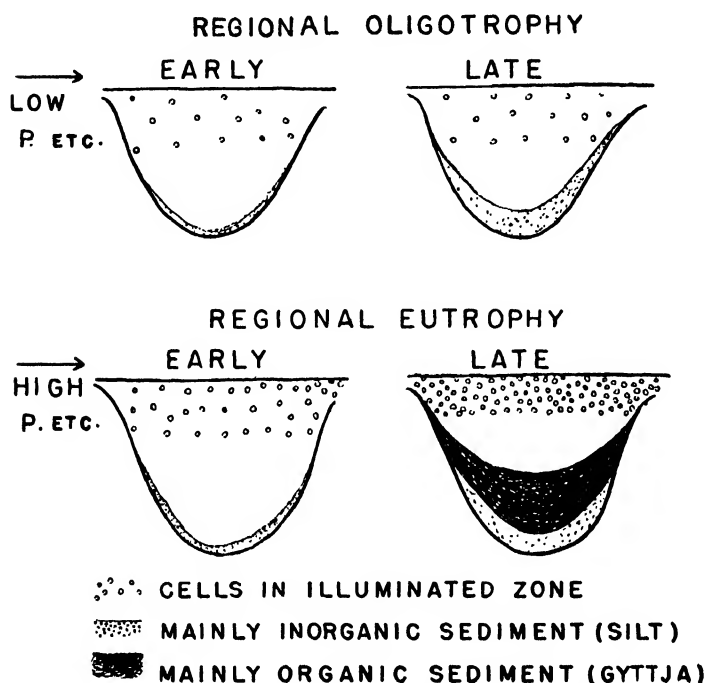


FIGURE 2. Ideal diagram of early and late stages in the development of a lake in an oligotrophic or nutrient-poor region, and in a eutrophic or nutrient-rich region.

occurred (FIGURE 3). This steady-state condition was evidently terminated by settlement in the eighteenth century, accompanied by deforestation, soil erosion, and rapid, complex changes in the lake. Subsequent to our studies, Pennington (1943; Jenkin, Mortimer, and Pennington, 1941) has obtained evidence of the same type of development in Windermere, in the English Lake District. In Pennington's profiles, the steady state is much more regularly maintained than in Linsley Pond, no doubt owing to the much greater size of Windermere and the consequent stability of the biological community in the face of external disturbances such as drought or forest fires. Pennington adopted an absolute time scale based on sedimentation rate within the lake, and this scale has been confirmed by Pearsall's (1946) recognition of two levels of increase in grass pollen as corresponding to Neolithic settlement about 1100 B.C. and Norse settlement between 1200 and 1400 A.D. Extrapolation beyond the verified part of the time scale gives the duration of the steady-state period as about 6000 years. Eastern North American pollen profiles cannot yet be referred to an absolute chronol-

ogy, but there can be little doubt that the steady-state period in Linsley Pond lasted several thousand years, too.

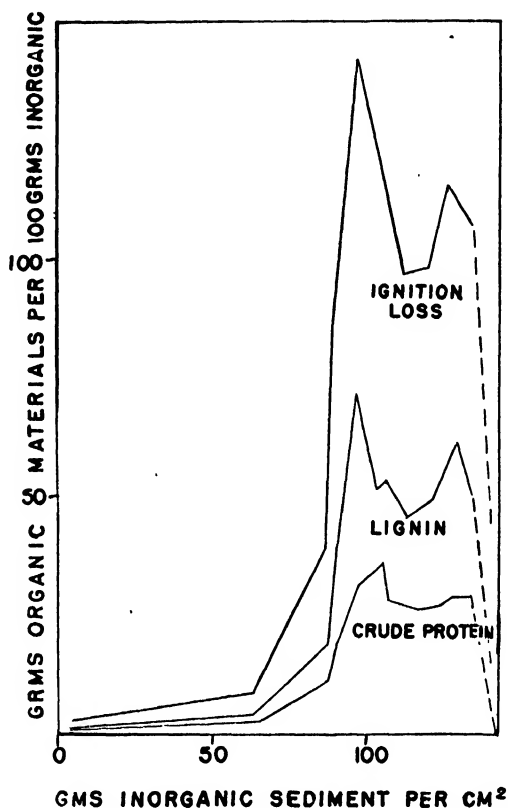


FIGURE 3. Ignitable matter, lignin and crude protein per gram of inorganic matter, as measures of organic production, plotted against total accumulation of inorganic sediment, as a measure of time. The final decline in all three curves is undoubtedly due to silt brought in at an abnormal rate after clearance and cultivation of the lake basin.

The steady-state period is recognized by the essential constancy in composition of its sediments. This is taken to mean that the productivity of the lake per unit area does not vary. Meanwhile, the lake is filling up with sediment, and the volume is therefore diminishing. Any properties of the lake dependent on the ratio of productivity to volume will, consequently, change. The volume of deep water cut off from circulation during the summer months will decrease, but not the organic matter that falls into it. The oxygen lost in a summer from unit volume of such water will therefore increase, and this progressive change will greatly change the qualitative aspects of the fauna. From a study of the arthropod remains fossilized in the mud, Deevey (1942) has beautifully shown how this happened in Linsley Pond. To the taxonomic biologist, the lake

early in the steady-state period would have seemed an entirely different locality from the lake at a late stage.

If the organic matter of the sediments may be used as a measure of productivity, it appears, therefore, that the variation of productivity with time can be expressed by a curve not unlike the sigmoid curve of growth, but that once the nutrient potential is being used with maximum efficiency, a number of purely qualitative changes can take place without altering the overall quantitative picture. The general mechanism of the steady state is comparable to the large self-regulatory cycles of the biosphere, the growth of the system being comparable to the growth of homogeneous populations to be discussed below. It is tempting to speculate as to whether, originally, living matter did not increase according to a like sigmoid law. After such a rapid expansion, vast qualitative changes with little quantitative variation in the total size of the system may have taken place.

## THE BIODEMOGRAPHIC APPROACH

### *Unlimited and Restricted Growth*

The unrestricted growth of a population,  $N$ , with a zero death-rate, such as a protozoan or bacterial culture dividing under constant environmental conditions, will be represented by the differential equation

$$\frac{dN}{dt} = Nb,$$

where  $b$  is the reproductive rate. For higher metazoa, the same law would be obeyed, if we regard  $b$  as the effective reproductive rate or difference between birth- and death-rate. In practice, some limit is obviously always imposed on this Malthusian expansion. It is usually assumed that the effect of the limiting conditions will tend to operate feebly but with an increasing effectiveness from the beginning of the expansion. The simplest expression of this situation is obtained by supposing  $b$  to be multiplied by a factor which measures the proportion of space still remaining for the population to occupy. If the saturation value be  $K$ , then

$$\frac{dN}{dt} = Nb \frac{K-N}{K}.$$

This is the derivative of the familiar Verhulst-Pearl logistic or sigmoid curve of growth. It is usually closely approximated in the growth of experimental populations that can be maintained under constant conditions, and for the study of such populations the use of the equation is fully justified in practice, even though it is hardly possible, in most cases, to decide between the logistic and certain other more complex expressions that have been proposed.

The Verhulst-Pearl logistic is not only applicable to populations. It

describes the growth of many single organisms or their parts. It is usual to regard organisms and their parts as growing like populations of protozoa or bacteria, by cell division. This, however, is clearly not necessary in order to produce a growth curve of the sigmoid form implied by the logistic, because in many cases most of the cell division occurs early in the process, while the later stages of growth occur solely through increase in cell size.

It has been indicated in the previous section of the paper that a qualitatively similar growth curve applies to the whole biocoenosis of a lake. In this case, we have no necessary organic continuity between the organisms of the biocoenosis early and late in the process. As has been indicated in detail, early colonizations insure that the geochemically determined trophic potential is used with increasing efficiency by later organisms, until a maximum efficiency is reached. It will be obvious that this represents a process formally analogous to, though biologically distinct from, the reproduction of the members of a population increasing towards a maximum defined by the trophic potential. The same analogy, but a different distinction in mechanism, is implied when sigmoid growth curves occur in economics (Lotka, 1925).

It is legitimate to regard the term  $\frac{K-N}{K}$  as formally describing a self-regulatory mechanism. There can be little doubt that, biologically, the mechanism can take a great variety of forms. The only formal condition that must be imposed on the biologically possible mechanisms is that they operate so rapidly that the lag,  $T$ , is negligible between  $t$  when any given value of  $N$  is reached, and the establishment of the appropriately corrected value of the effective reproductive rate  $b \frac{K-N}{K}$ .

If there is a time lag  $\tau$  such that the rate  $\frac{dN}{dt}$  at time  $t$  is determined by  $N(t-\tau)$ , then oscillations will be introduced into the system. The growth curve will pass the saturation level  $K$  at, e.g.,  $t_1$ , and then at time  $t_1+\tau$  the rate of change of the population will become zero. The population will then decline, reaching  $K$ , now from above, at  $t_2$ , and again at  $t_2+\tau$  the rate will become zero and the curve will then approach  $K$  from below. Further work is needed in the analysis of this case,\* but it is evident that the longer  $T$ , the more will  $N$  surpass the saturation value in the first ascent of the curve. For small values  $T$ , the oscillations will die out and the saturation value will be approached.

\* Professor Lars Onsager has kindly examined the mathematical aspect of this situation. Writing  $y = N/K$  and measuring time in terms of the interval required to make  $b$  equal to unity, the equation for a time lag  $\tau$  can be written  $\frac{dy}{dt} = y_t(1 - y_{t-\tau})$ . It can be shown that if  $\tau < \frac{\pi}{2}$ , the oscillations tend to die out. If  $\tau > \frac{\pi}{2}$ , the oscillations tend to increase but are limited by the condition  $0 < y < e^\tau$ . The areas between  $y=1$  and the curve are equal (though of opposite sign when measured with reference to  $y=1$ ) for successive half periods. When  $\tau$  is large, this means that a succession of high peaks alternate with very wide troughs, producing a biologically unstable situation in which short periods of excessive overcrowding alternate with long periods in which the population is dangerously rarefied.

It is quite likely that a number of cases of this phenomenon will be discovered. Pratt (1943) has shown that, in *Daphnia* populations, oscillations can be set up which are in part due to the fact that the fertility of a parthenogenetic female is determined not merely by the population density at a given time but also by the past densities to which it has been exposed. Much recent work on rodent populations reviewed by Elton (1942) and by Errington (1946) indicates that at high densities a great deal of intraspecific fighting may take place. Younger animals about to embark on their reproductive career are more likely to succumb in such fighting than are old experienced animals whose remaining reproductive career is short. The net result of fighting at high density is to change the age composition of the population in favor of old non-reproductive individuals, which soon die off from other causes. In nature, the events are not continuous, but dependent on a seasonal cycle which probably enhances oscillations generated by the purely internal demographic factors. It is quite possible that phenomena of this sort, which, in effect, involve the operation, with a time lag, of population density on net rate of increase, play a part in generating the well-known cyclic changes in the populations of field mice. Though it is probable that many more cases of the operation of time lags could be found, it seems likely, from the number of certain cases where they do not operate, that the phenomenon is relatively rare. It is highly probable that there is a general tendency for the time lag to be reduced as much as possible by natural selection. Supersaturated values of the population may introduce new unfavorable conditions, causing epidemics or other disasters, while if this does not happen the population is likely to be exposed to greater risk of extermination by chance unfavorable factors at the times of the subsequent minima in the oscillation. In spite of some glaring exceptions, it seems probable that an internally oscillating population is less likely to survive indefinitely than a stable one. If this be so, the time lags will be reduced to minimal values.

*Competition and the Balance of Nature.* Volterra (1926) and Gause (1934-1935) have examined what happens when two populations are competing for a food supply maintained at a constant level

$$\frac{dN_1}{dt} = N_1 b_1 \left( \frac{K_1 - N_1 - \alpha N_2}{K_1} \right)$$

$$\frac{dN_2}{dt} = N_2 b_2 \left( \frac{K_2 - N_2 - \beta N_1}{K_2} \right),$$

where  $\alpha$  and  $\beta$  are coefficients of competition, indicating the depressive effect of a unit of one population on a unit of the other. Negative values of both  $\alpha$  and  $\beta$  correspond to symbiosis, a positive value of one and a negative value of the other to certain kinds of commensalism and parasitism (Gause and Witt, 1935). When mutual competition takes place, both coefficients being positive, three cases may be distinguished:

$$\alpha > \frac{K_1}{K_2}, \beta > \frac{K_2}{K_1} \quad (1)$$

The members of each species act unfavorably on the members of the other, more powerfully than they do on the members of their own species. The final result is that only one species is left, and the species to survive is determined by the initial proportion. Gause had no example of this phenomenon, but it could probably be realized in cultures of microorganisms producing mutually inhibitory antibiotics.

$$\alpha > \frac{K_1}{K_2}, \beta < \frac{K_2}{K_1}; \text{ or } \alpha < \frac{K_1}{K_2}, \beta > \frac{K_2}{K_1} \quad (2)$$

Competition takes place, and the final result, whatever the initial condition, is determined by the relative values of the coefficients. This case has been generalized for  $n$  species by Volterra.

$$\alpha < \frac{K_1}{K_2}, \beta < \frac{K_2}{K_1} \quad (3)$$

Both species survive indefinitely at an equilibrium concentration.

When competition for food is involved, it is obvious that (3) can be realized if a system be set up in which competition could lead to displacement of species 1 by species 2 and at the same time a volume of space, termed a *refuge* by Gause, be provided into which species 2 cannot go. More generally, if there are two spatially defined regions, in one of which  $\alpha > \frac{K_1}{K_2}, \beta < \frac{K_2}{K_1}$  and in the other  $\alpha < \frac{K_1}{K_2}, \beta > \frac{K_2}{K_1}$ , then the conditions of (3) can be realized and the species can coexist. Following the usual terminology, the two species are said to have separate though partly overlapping niches, and so long as this is the case they can persist indefinitely. In general, wherever in nature two species coexist in a region and feed on the same food, they will be found to have slightly different ranges of environmental requirements. It is commonly recognized by ecologists that closely allied species living together practically always occupy slightly different niches or, in other words, have different tolerances and optima. Two possible exceptions to this rule may be expected. (1) Where some external factor acts to rarify the mixed population, so that the environmental possibilities are hardly exploited, the process of expulsion of one species by the other might never progress beyond the initial stages (*cf.* Crombie, 1945). A powerful and quite indiscriminate predator might maintain two species of prey at such a low level that they never come into effective competition. (2) Where the values of  $\alpha$  and  $\beta$  are under environmental control, as is indeed implied by the theory of niches, continual chance oscillations of the environmental variables might continually reverse the direction of competition, so that no equilibrium could ever be established. The numbers of the competition vary

irregularly, but all are always present. It has been suggested that this situation is exemplified by mixed phytoplankton populations in lakes (Hutchinson, 1944b).

*Prey-Predator Relationships.* The most striking theoretical results involving biodemographic circular causality are probably those of Lotka (1925), Volterra (1926), and other investigators on prey-predator and host-parasite relationships. The simplest case, considered by Volterra, may be developed as follows:

If  $N_1$  be the number of prey and  $N_2$  the number of predators, and predation is taken to be proportional to the number of random encounters, then, if the populations are sufficiently sparse to permit neglecting the regulatory mechanism discussed in the previous section,

$$\frac{dN_1}{dt} = N_1 (b_1 - p_1 N_2)$$

$$\frac{dN_2}{dt} = N_2 (-d_2 + p_2 N_1)$$

where  $b_1$  is the effective birth-rate of the prey in the absence of predation,  $d_2$  the effective death-rate of predator in the absence of food, and  $p_1$  and  $p_2$  coefficients expressing the effect of predation on the two populations. The solution of this pair of simultaneous differential equations is

$$\left( \frac{p_2 N_1}{d_2 e^{\frac{p_2 N_1}{d_2}}} \right)^{d_2} = C \left( \frac{p_1 N_2}{b_1 e^{\frac{p_1 N_2}{b_1}}} \right)^{-b_1}$$

which, when  $N_1$  is plotted against  $N_2$ , gives a series of closed curves moving round the singular point

$$N_1 = \frac{d_2}{p_2}, \quad N_2 = \frac{b_1}{p_1}$$

It is important to note that  $p_2$ , the efficiency with which individuals of prey are converted into predator, depends on  $p_1$ , the efficiency with which predators kill prey. It is probably not unreasonable to write  $kp_1$  for  $p_2$ . Thus, the greater the likelihood that death of prey follows encounters, the closer the singular point is to the origin. Gause has found experimentally that if the predation rate be maintained artificially low by continual rarification of the predators, then the cyclical variation postulated by Volterra does take place, but that in a confined system with predatory microorganisms feeding at their natural rate, the values of the predation coefficients are so great that oscillation around the singular point becomes statistically impossible. In other cases, other, more complex, situations, which in effect raise the predation rate, may develop. In nature, the only cases where it seems likely that typical Lotka-Volterra oscillations develop is where organisms live in a rather dense medium, such as soil or flour, which impedes free movement. The

classical cases of persistent oscillations, such as the snowshoe rabbit, *Lepus americanus*, and the lynx, *Lynx canadensis*, are unlikely to be due to the prey-predator relationship, because they seem to occur in a prey population in the absence of the predator.

*The Classification of Periodicities in Populations.* Some of the material that has been presented above may provide a useful scheme for the clarification of cyclical phenomena in populations. It is desirable, first, to point out that in a great many cases of fluctuation in animal populations where periodicities have allegedly been detected, they are probably of a spurious nature.

If some environmental variable,  $x$ , fluctuates over a range of values, and  $p$  is the probability of values in excess of a certain given value,  $k$ , and if the occurrence of a certain biological event be implied by the condition  $x \geq k$ , then it is clear that  $p$  is the probability of the occurrence of the biological event. Now if  $p$  be taken as constant over a long time-span and if two parts of the time-span be compared, the frequency of occurrence of the biological event will be approximately the same in the two parts of the time-span. If, in such a case, the biological system has an autoregressive property, so that its state depends on all previous states, as well as on the value of  $x$  at the time under consideration, the variation of the biological system will be smoothed, often showing quasi-periodic movements of low persistence, and also occasional great irregular increases or decreases, reminiscent of the irregularly occurring years of excessive abundance or scarcity so often encountered in nature.\* It seems not unlikely that the supposed cycles with a 5- to 6-year period in British game animals described by Middleton (1934) are, in part, of this nature.

Rejecting the statistical quasi-periodicities of this sort, we may, theoretically, distinguish cases in which the periodicity is *extrinsic* from those in which it is *intrinsic*. The valid cases of extrinsic periodicities are mainly determined by diurnal, lunar, tidal, or annual periods; in other words, by the mechanical properties of the solar system. There can be little doubt that there often are solar rhythms corresponding to the sun-spot cycles observable in tree growth, but the evidence that such rhythms are reflected in the variations of animal populations is totally inadequate. A few cases in which fairly regular extrinsic periodicities are imposed on populations by periodic meteorological phenomena have been described. The best case is probably the variation of the population of the guanay *Phalacrocorax bougainvillei* on the Peruvian coast in response to abnormal hydrography, seemingly with a period of about seven years. This case will be critically discussed in detail in a later publication.

\* A convenient model may be made by taking for  $x$  the values of a successive series of throws of two dice. The extreme conditions (2, 12) here appear more rarely than the intermediates. If the population  $P_n$  at time  $t_n$  is conceived as dependent on  $P_{n-1}$  and on a suitable function of  $x_n$ , a curve that is very like the more irregular type of population density curve often encountered in field studies can be obtained.



Intrinsic periodicities are presumably best divided into cases in which the factors producing a decline are operative throughout the cycle, as in the case of predation, or those in which they only operate at abnormal densities, as in pandemic disease. Where the operation is continuous, there is an obvious dichotomy between the interaction of a population with itself at a later time and the interaction of two populations of different species. Finally, there are cases in which the periodicity is intrinsic to one species but entirely extrinsic to others. The classification may be expressed as follows:

I. Extrinsic to biological community.

- (a) Planetary mechanical, *e.g.*, diurnal, tidal, lunar, or annual.
- (b) Solar (very doubtful).
- (c) Other meteorologically determined cycles.

II. Intrinsic to biological community.

- (a) Mechanism of decline discontinuous.
- (b) Mechanism of decline continuous;
  - (1) Interaction within a species,
  - (2) Interaction of more than one species.
- (c) Derived periodicity extrinsic to species under consideration, intrinsic to another species.

*Oscillation and Natural Selection.* The theoretical approach to circular causal systems in ecology suggests that these oscillating systems should be found frequently in nature. Certain oscillations have been discovered and have been investigated extensively, though little understanding of their nature has been achieved. Practically no cases can as yet be placed in their proper categories in the theoretical scheme. The great attention that has been paid to the few good examples, notably to the periodicity of microtine rodents and of Canadian fur-bearing mammals, is probably in part a reflection of the highly exceptional nature of such occurrences. They are best regarded, whatever their mechanisms, as derangements of self-regulatory systems which in highly exceptional circumstances have been able to survive. More usually, such oscillations would be expected to lead to catastrophe, and the species exhibiting them would disappear. It has already been hinted that natural selection may have reduced the time lags which can give rise to interaction within a single species. The same situation may be reflected in the highly artificial conditions needed to realize the Lotka-Volterra cycle in the laboratory. It seems quite possible that a number of other cases will be discovered in which there is an inherent probability that perfecting of self-regulatory mechanisms has occurred, leading to a decreased chance of oscillation. The characters of species involved in such evolution would be characters exhibited only in populations, and the selection would be of a kind that involved replacement of one population by an adjacent and initially partly isolated population. Selection of this type is of course commonly recognized in modern evolutionary theory.

*The Concept of Saturation.* The sigmoid growth curve has been shown to apply, notably by Lotka (1925), to a number of situations in the de-

velopment of human culture. It is clear that a given population can be saturated with certain commodities, and if, as is usually the case with durable goods, the potential "birth-rate" exceeds the "death-rate," the effective "birth-rate" tends to zero as  $K$  is approached and unemployment results. The regulation of the "birth-rate" leads to immediate privation, and in nearly saturated capitalist communities it has seemed preferable to introduce an artificial "death-rate," insuring that commodities do not last as long as they might, in order to maintain an appreciable rate of production. This process ensures a long-term privation owing to an excessive rate of depletion of natural resources. When attention is diverted from material culture to non-material culture, two situations apparently are possible. Kroeber (1944) has indicated how particular styles in art or particular fields of thought may reach saturation. The great periods in art or thought are apparently those where the rate of development of the style or subject of inquiry is greatest. Around the time of maximum  $\frac{dN}{dt}$ , a given individual can do more in a normal lifetime than at any other period. When  $K$  has been achieved, we can look back on the whole process, appreciating and enjoying the entire body of work but the great names, the geni, are associated with a maximum value of  $\frac{dN}{dt}$ , and the inspiration of the period thus seems to rise and fall.

When we look at the whole of human knowledge, however, it seems to grow indefinitely. Actually, such indefinite growth is probably a characteristic only of certain cultures. In other cultures, new adaptations and new ideas may appear and replace old ones, but no progressive increase in the total number of elements in the non-material culture need appear. Vernadsky has introduced the term, *corpus scientiarum*, for the ever-expanding body of organized knowledge that seems unlimited by a saturation value. It may be composed, as Kroeber\* evidently feels, of numerous saturating systems, but the number of these saturating systems evidently continues to increase. Lotka (1946), using the number of pages in Darmstaedter's *Handbuch der Geschichte der Naturwissenschaften und der Technik*, devoted to inventions and discoveries dating from each successive century, has drawn a graph which, though admittedly in part based on elements of material culture, is derived from elements which are likely to increase with the *corpus scientiarum*. In FIGURE 4, his graph is replotted to give the logarithm of his particular function of the *corpus scientiarum* against time. Here it is to be noted that the birth-rate actually increases with time. This is what one would expect, because, as Mr. Gregory Bateson has pointed out,† the birth-rate depends in part on the number of possible relations between the elements composing the population and not on their number. This type of growth is an entirely new phenomenon in the history of the earth. However des-

\* Personal communication.

† Personal conversation.

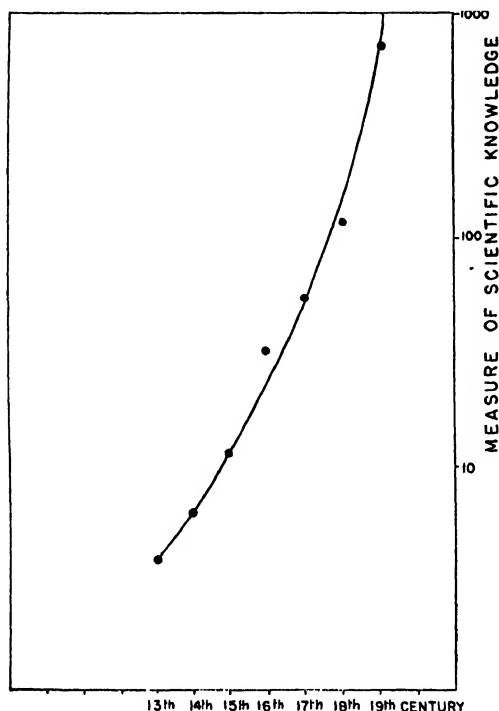


FIGURE 4. The logarithm of Lotka's measure of the *corpus scientiarum*, plotted against time.

perate the situation may be into which mankind has brought itself, the development of this new kind of growth gives a reason for hope, a reminder that on looking back the seemingly impossible appears to have been transcended, and that in looking forward the path, however difficult, need not again prove impassible.

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# THE VICIOUS CIRCLE IN CAUSALGIA

By W. K. LIVINGSTON

*Department of Surgery, University of Oregon Medical School,  
Portland, Oregon*

IN 1872, Dr. S. Weir Mitchell<sup>1</sup> published a book entitled *Nerve Injuries and their Consequences*, in which he reported his observations and opinions based on a study of a considerable number of soldiers who had sustained nerve injuries during the battles of the Civil War. Among these men, there were a few whose complaints of pain were so excessive, and whose physical signs were so diffuse and bizarre, as to make it difficult to believe that they could be caused by the associated nerve lesions. Casualties complained of an extreme degree of burning pain in the wounded limb. When the hand was affected, the pain was worst in the palm, while with the foot involved, the pain was worse on its dorsum. The part might be so sensitive that the patient kept it wrapped in oiled silk or wet cloth, to protect it from any kind of stimulation, even a breath of air. Some of the men insisted that the skin over their entire body had become sensitized to such an extent that dry contact or skin friction caused sharp shocks of pain to shoot into the affected limb. They seemed to get some degree of relief by keeping the limb constantly moistened with water, and by keeping their hands continually wet. Some of the men even poured water into their boots. Examination of the men so afflicted showed very striking changes to be taking place in the tissues of the limb. The fingernails were arched and ridged; the skin was usually red and glossy, as if varnished, or macerated and odoriferous from long immersion in water; the muscles were wasted and incoordinate; the joints were stiffened; and in the well-advanced cases, the whole function of a hand might be permanently lost. In addition to this physical disability, the continued pain and hyperesthesia tended to undermine the patients' morale.

Mitchell accepted these cases as real, and attributed their troubles to the nerve wound. He was particularly impressed by the burning character of the pain in some of his cases, and he described it as "the most terrible of all the tortures which a nerve wound may inflict." He gave it the name "causalgia," which is derived from the Greek words meaning "burning pain." His descriptions of the pain, hyperesthesia, and trophic changes observed in his wounded patients represent some of the most dramatic clinical pictures in the entire medical literature.

Today, the term "causalgia" is not used, as I believe Mitchell employed it, to indicate a certain kind of pain, but to designate a clinical syndrome characterized by burning pain, glossy skin, and hyperthermia. Although each of these features occurs frequently as a result of chronic nerve irritation, it is most unusual to find them all combined in one case.

It is unfortunate that the term "causalgia" should be restricted to this rare combination. And it is also unfortunate that Mitchell's descriptions were so dramatic, because many physicians refuse to apply the term "causalgia" to any case which does not combine all of the features cited in the degree of severity exhibited by the worst of Mitchell's cases. I have talked with men who examined large numbers of nerve casualty cases in World War II, and who tell me that they have never seen a case of causalgia. On the other hand, there are occasional reports dealing with large numbers of cases, perhaps a hundred or more, all classed as instances of causalgia, and all treated by the same physician. Obviously, such discrepancy must rest upon an interpretation of terminology. It might be better if we applied "syndrome of nerve irritation" to the extensive group of cases with irritative nerve lesions, and reserved the designation "causalgia" for the more severe types of this syndrome in which burning pain is a prominent feature.

Since nerves are the conductors of the impulses subserving the sensation of pain, it might be assumed that all nerve injuries would be painful. One might assume, further, that the more extensive the nerve injury, the more likely it would be to cause severe pain. As a matter of fact neither of these assumptions is justified. In the great majority of nerve casualties which I have treated, there has been no more suffering than would be occasioned by an equally extensive wound that spared the major nerves. Most of my patients complained of little or no pain from the moment of wounding to the completion of their convalescence.

Nor is there any reliable parallel between the extent of the nerve injury and the mischief it is capable of causing. No one has yet been able to point out the essential nature of the pathology responsible for the syndrome of nerve irritation. Some of the worst cases of causalgia have resulted from a relatively innocuous-appearing partial lesion of a major nerve. At the Oakland Naval Hospital, we have carefully studied more than 1,200 nerve casualty cases. Among these, there were only 133 men (11 per cent of the total) who complained of burning pain. In approximately half of these, the pain was never said to be excessive, and it tended to subside spontaneously. In 58 cases, the burning pain was the presenting symptom and required special attention. In 33 of these (2.66%), the signs and symptoms were sufficiently severe to warrant the designation "causalgia."

*Case Report.* L.H.B., a 26-year-old marine, was wounded above the posterior aspect of his right knee on April 26, 1945, during the Okinawa campaign. His platoon was moving slowly forward in the rain, along a ridge. The men were strung out in a line. A machine gun suddenly opened up from a Japanese ambush on their right flank. B. heard the firing and saw men fall as the fire swept back along the line. He ran for a hole but was hit in the right leg just before he reached it. He dived into the hole with his right leg dragging uselessly behind him. He was in no

pain as he crouched in the hole. He recalls that his right foot felt "warm," while the rest of his body was wet and miserably cold. He received morphine and had his wound dressed within fifteen minutes of the wounding. He was taken by stretcher to the company aid station, and from there by jeep to an army hospital and thence to a hospital ship.

He became aware of a burning, tingling sensation in his right foot during the second day. The pain steadily increased until it was said to be "unbearable." His reactions to pain were so extreme that his attending physicians thought him to be "hysterical." This interpretation of his pain was so much emphasized to the patient that when he entered the Oakland Naval Hospital, he volunteered that "the pain was probably all in his head" and then refused to discuss it further. His right foot was red and swollen. The skin showed several superficial ulcerations, and between them, masses of macerated and flaking skin piled up. The foot was so sensitive that the patient would permit no one to touch it and would break into sobs if anyone asked to examine it or even made a quick move toward it. There was some shortening of the calf muscles; yet it was obvious that they functioned, because he would occasionally walk around during the night. If the ward was very quiet, he might moisten his foot with water and slip on a loose-fitting, thick-soled tennis shoe and limp around the ward for a time. At one period, we tried forcing him to take some exercise during the day. He insisted on using his crutches, and would hobble up and down the ward crying loudly, subsiding only when he got back on the bed with his foot in his pan of water. Almost any kind of sensory stimulus seemed to make his pain worse. Things that he saw or heard, particularly if they caused a startle reaction or had an emotional response, were able to aggravate the pain. Seeing another patient walk with crutches on a polished floor made his foot hurt worse. Sudden noises, the sound of airplane propellers, and particularly the scraping of a shoe on the deck caused sharp shocks of pain in the foot. Contact stimuli, not only to the affected foot but to the skin of normal parts of his body, were objectionable. Sliding his normal left foot down between the sheets, or putting it bare on the deck, were said to cause extreme pain in the patient's other foot. He was unable to turn the pages of a book or touch anything when his fingers were dry. He constantly kept a moist washcloth in his hands and carefully moistened his fingers before grasping anything. If the object he was holding started to slip from his grasp so as to cause skin friction, he promptly dropped it, no matter what it might be.

This man was given a variety of novocaine injections and other forms of treatment, none of which afforded any lasting relief. While he had been on the hospital ship, he had been given a novocaine block of his lumbar sympathetic ganglia. Although he admitted that the injection gave him considerable relief for several days, he had developed an inordinate fear of the procedure and for a long time steadfastly refused to permit us to repeat it. He submitted without resistance to an explora-



tion of the wound area. The sciatic nerve was found to be enlarged and irregular and in one portion was adherent to the scar tract left by the bullet. Yet electrical stimulation of the nerve demonstrated good muscle responses in both leg compartments. We were tempted to resect the sciatic nerve in the hope of relieving his pain, but were reluctant to sacrifice its function. A simple neurolysis and covering of the lesion with a tantalum sleeve brought about only a slight relief of pain and produced no change in his hyperesthesia. Finally, he agreed to submit to another block of his sympathetic ganglia. The novocaine injection was followed by an equal amount of alcohol. The patient experienced complete and persistent relief following this injection. Within two days, he was wearing shoes and walking everywhere. The shortening of the calf muscles prevented him from walking normally for a few weeks; otherwise, the improvement in his physical and emotional status was dramatic in its suddenness. He was discharged to duty eleven months from the time of his wounding.

Most of our patients were more stoical in their reaction to pain than this man, yet careful observation left no doubt that they suffered intense pain. In some of them, the trophic changes in the affected foot or hand were more marked, and there were instances in which a hand remained stiff and seriously disabled long after the pain and hyperesthesia had disappeared. The trophic changes that may develop in causalgia are of considerable interest, since they involve all of the tissues of the limb and hardly ever remain confined to the distribution of the affected nerve. In our cases, it was evident from the circulatory changes, the muscle dysfunction, and the excessive sweating that some profound abnormality in the local physiology was present. If the muscles could be used at all, their movements were weak and incoordinate, and often an irregular jerking of individual fingers was present. These movements were too coarse and irregular to be classed as a tremor. We termed them "cog-wheel movements," although a better designation would be "muscle stammering." If sweating was present at all, it was likely to be excessive. I have seen a case in which sweat dripped continuously from a single finger. In one of our cases, whose affected foot was warm and dry, it was the contralateral foot which exhibited the excessive sweat reactions. Under ordinary circumstances, this normal foot did not sweat any more than the rest of the patient's sweat areas. However, when the affected foot was lightly rubbed, or when an attempt was made to straighten out the patient's knee contracture, the contralateral foot would break out in droplets of sweat. If the painful stimulation was continued, the droplets would coalesce to form rivulets of sweat which would roll off the foot to soak the sheets.

*Treatment*

The problem of treating causalgia imposes a considerable burden on the physician, particularly if he is sympathetic toward his patient's suffering. We have used a wide variety of treatments, all of them meeting with some degree of success. The most reliable treatment is that of sympathetic ganglionectomy. It is most likely to succeed when it is done early. Some surgeons have been so impressed by the dramatic improvement that frequently follows this procedure, that they consider causalgia to be a "sympathetic dystrophy." A few observers believe that sympathetic pathways conduct pain impulses from the extremities. They attribute the cures obtained by ganglionectomy to a simple interruption of a pain pathway. There is very little experimental evidence to support this contention. Also, sympathectomy too often fails to justify the conclusion that the activities of the sympathetic system are essential to the maintenance of the causalgic state. The sympathetics are evidently but one element in the vicious circle that maintains the syndrome. I am always reluctant to sacrifice the ganglionic chain if simpler methods can attain a satisfactory result. Moreover, if sympathectomy fails to relieve the condition, I feel that the surgeon has lost his "ace in the hole," in having excised a pathway at which the syndrome is most vulnerable to attack. In our series of 33 cases, there are still four or five men who have traces of their original pain. It may be significant that three of them had been subjected to sympathectomy.

When asked what treatment I would recommend for a well-established syndrome of causalgia, I am at a loss to know how to answer. I am inclined to say that the course of treatment is much like the progress of a chess game. The physician makes a move, and the response of his patient will determine his subsequent move. The final checkmate is likely to be the result of an intelligent campaign rather than of a single brilliant move. However, there are two general principles which should be followed whenever it is possible to do so: "Treat the pain early, and establish full use of the extremity as soon as possible." I am convinced that when pain can be controlled soon after its onset, whether by medication, novocaine blocks, or the more radical sympathectomy, the full syndrome of causalgia will not develop. Sometimes the simple covering of an exposed nerve or a neurolysis will abort the process. And I have learned that if the patient can tolerate the pain which early use of the extremity causes, he is already well on the road to spontaneous recovery.

Unfortunately, in many cases the condition is not recognized early, and the patient reaches the physician with the syndrome well developed. It is not possible for the patient to use his hand when pain and hyperesthesia are severe. Since almost anything he does with the limb increases his terrible pain, he retreats from all activity. His very posture denotes a "cradling" protection of the hand from all contact. He simply cannot tolerate the usual forms of physiotherapy, such as heat and massage. It

may be possible to start treatment in the whirlpool bath, beginning with the slightest agitation of the water. If this does not cause too much pain, it may be possible gradually to increase the water friction, then to institute underwater massage and manipulation, and finally to progress to the point where these forms of treatment can be employed out of the water. Under this regime, one observes a gradual recession of the pain and hyperesthesia; the circulation improves, and the abnormal tissue changes begin to disappear. If the patient can be brought to the point where he will begin making active use of his affected hand or foot, we are almost certain that he will continue to improve spontaneously. We have been so much impressed by the beneficial effects of active use that we have concluded that the inflow of sensory impulses it initiates, must act to condition the activities of the spinal-cord centers. We have come to feel that the more normal the input of sensory impulses, the more likely it is that the output from the regulatory centers to the limb will be "normal." We have expressed this hypothetical concept by saying, "If you wish a normal output, you must first establish the conditions which will permit a normal input."

As a means of reducing pain and assisting the patient in submitting to this regime, we have found that intermittent novocaine block of nerve impulses is of considerable assistance. Novocaine block of the affected nerve, either proximal or distal to the lesion, often materially reduces pain for hours or days. An even more striking and persistent relief may be effected by a block of the sympathetic pathways. Sometimes, a novocaine block changes the character, extent, or degree of the pain permanently, and occasionally a single injection abolishes the syndrome. This is a most puzzling observation, since nothing that we know about the local action of novocaine would lead us to anticipate any such result. The pharmacology of this drug is well understood. In the solutions employed in the clinic, it does not destroy nerve fibers. It acts as a local anesthetic which, for a period of an hour or two, blocks conduction in the nerve fibers. Then it is gradually washed away by the circulation, and the fibers resume their usual ability to transmit impulses. When a novocaine injection of the sympathetic ganglia succeeds in reducing or abolishing the pain in one of my causalgia cases, I know that the drug has not permanently interrupted the sympathetic pathway, because sweating and both pilomotor and vasomotor reflexes are present as soon as the local effect of the novocaine wears off. Yet in spite of its apparently transient effects, it does something which can profoundly alter the syndrome of causalgia.

I do not wish to leave the impression that novocaine injections can be relied upon to cure or even alleviate causalgia in all cases. Indeed, it fails more often than it cures. Yet the fact that it sometimes abolishes the syndrome of causalgia is an arresting fact, and its few cures may be of greater significance than its many failures. That this fact is not a coincidence is indicated by the many reports from other clinics in which

a novocaine injection has abolished pain syndromes of long standing. Because I believe that these facts are significant, I would like to digress for a moment to discuss briefly two other types of pain syndrome in which novocaine block has produced unexpected results.

*Phantom Limb Pain.* Following the amputation of a limb, it is usual for the subject to experience sensations which are ascribed to the absent part. These sensations, which are usually most vivid immediately after the amputation, tend to fade as time passes. Yet not infrequently, instead of fading they tend to increase, until they may become so distracting to the patient that he loses his social usefulness. Many of the sensations are painful, and it may very well be that it is the pain which acts to maintain the image of the phantom limb so vividly in the patient's mind. The pain may have the same burning quality that characterizes causalgia, or it may be "boring," "tearing," or "cramping." Usually the phantom hand is felt to be held in a fixed immovable posture, from which the patient is unable to move it by any effort of his will. The sense of tension is sometimes as objectionable to the patient as the more severe pains. The pains are rarely ascribable to any obvious fault in the stump, and re-amputation at a higher level usually fails to cure the pain syndrome. In some cases, the muscles of the stump may exhibit clonic contractions at times, or jerk unexpectedly.

Sometimes, the stump is cold, discolored and sensitive, and not infrequently there is excessive sweating from the axilla of the affected side. I have examined an amputee who sweated normally on the intact side, while on the side of his amputation the sweat used to soak his shirt and the top of his trousers.

In a case of this kind, it sometimes happens that a novocaine injection of the appropriate sympathetic ganglia will produce a most astonishing sequence of events. The patient usually reports, first, that his phantom hand seems to be getting warm and relaxing its tension. Then, one after the other his phantom fingers, which for years may have been immovable, open and can be voluntarily moved. The pain in the phantom limb disappears; the stump warms and its sensitiveness disappears. In the majority of cases, these changes are temporary, and within a few hours or days, the pains recur and the phantom hand returns to its fixed posture. Yet in the exceptional case, the relief may be permanent. I have observed cases in which the pain did not recur for months or years. The most remarkable feature of this result of a single novocaine injection is the fact that when the pain does not recur, neither does the excessive sweating, nor the coldness, discoloration, and sensitiveness of the stump.

Another type of case in which novocaine injections produced effects that are difficult to account for, is the following: A stenographer of 38 was referred to me, some years ago, for investigation of headaches associated with an itching and burning sensation in the left side of her forehead and scalp. Eighteen months previously, she had been in an auto-

mobile accident, in which she sustained a fractured femur, a fractured jaw, and a deep laceration above her left eye. For about six months after the accident, the left side of her forehead and scalp felt "numb." When sensation began to return to this area, she experienced intermittent attacks of intolerable itching of the scalp. If she scratched it, a severe burning sensation supervened, which might last for hours, sometimes persisting all night. Coincident with the return of sensibility in the left side of her forehead, the patient began to suffer from headache. The ache always seemed to start over the left eye, then spread to the temples, and finally involved the whole head. In the year that had elapsed since their onset, the headaches had increased in severity and frequency, so that they had begun seriously to interfere with her work. In addition to the headache, she complained that she could not lie on her left side, because this brought on dizziness and a sensation as if she were whirling in space. Examination showed the scar over her left eye to be exquisitely sensitive, and there was a marked hyperesthesia over the distribution of the left supraorbital nerve. An injection of the scar with novocaine temporarily aggravated her complaints, followed by a few days in which she was free from the scalp itching and headache. In a period of one month, she was given four such injections, after which her symptoms did not recur.

The syndrome of nerve irritation, with causalgia as its most striking example, and the fact that a temporary interruption of nerve conduction by a local anesthetic may cure it, constitute a real challenge to the physiologist. Here are some of the questions which require an answer. Why are partial lesions of nerves more prone to precipitate the syndrome of nerve irritation than a complete transection of the nerve? Why do so few nerve casualties develop the syndrome? What is the essential nature of the lesion responsible for it? How can such a lesion act to give rise to pain and hyperesthesia, to muscle dysfunctions, to excess sweating and vasomotor disturbances, and eventually to trophic changes in the tissues? Why do the signs and symptoms tend to spread so far beyond the distribution of the affected nerve? How is it possible to develop sensitiveness of the skin of the entire body, expressing itself as pain confined to the affected limb? Why does novocaine block confer relief in so many cases, and why does the relief tend to outlast so greatly the local effects of the drug?

### *Hypothesis*

In my own attempts to answer some of the questions just mentioned, I have come to feel that the local lesion is merely the "trigger point," or inciting mechanism, which starts a vicious circle of reflexes. Once started, the process tends to become self-sustaining, so that even removal of the trigger point may not stop its activity. I know that the sympathetic system is a part of the circular causal mechanism, not only because of the obvious vasomotor and sudomotor disturbances, but be-

cause an interruption of the sympathetic pathways often cures the syndrome. On the other hand, I can be sure that the sympathetic influence is but one part of the process, since the syndrome can persist after the sympathetic pathways have been excised. I know, too, that the anterior horn cells must be involved in the central process, because of the alterations in status and function of the skeletal muscles, and I suspect that the extreme pain and hyperesthesia point toward an implication of the posterior horn cells in the same central perturbation. Thus, I come to visualize some agency in the spinal cord whose normal activities are augmented, and its perfect synchronizations disturbed, by sensory impulses from the trigger point. This agency must play on all three horns of gray matter in the spinal cord, so as to alter their function.

In its clinical form I would express this hypothetical concept as follows<sup>2</sup>: "An organic lesion at the periphery involving sensory filaments may become a source of chronic irritation. Afferent impulses from this 'trigger point' eventually create an abnormal state of activity in the internuncial centers of the spinal gray matter. The internuncial disturbance, in turn, is reflected in an abnormal motor response from both the lateral and anterior horn neurons of one or more segments of the cord. The muscle spasm, vasomotor changes, and other effects which this central perturbation of function brings about in the peripheral tissues, may furnish new sources for pain and new reflexes. A vicious circle of activity is created. If the trigger point is removed early, the process may subside spontaneously. If the disturbance is permitted to continue, it spreads to involve new areas, and tends to acquire a momentum that is increasingly difficult to displace. Perhaps at this stage, even a removal of the original irritant may not be sufficient to establish a cure. But if an important part of the circle of reflexes can be interrupted, the process may subside, and a normal physiological status is again established. If again the pathological patterns gain the ascendancy, the repeated breaking of the circle may result in a permanent cure."

### *Experimental Evidence*

It must be realized that this hypothetical formulation which I have advanced to account for the syndrome of nerve irritation and the effects sometimes produced by novocaine block, has never been confirmed by laboratory investigation. Yet there are three laboratory contributions which I wish to cite briefly, since I feel that they lend some support to the concept. The first of these deals with the local lesion, the second with the nature of the central agency, and the third with the phenomenon known as "facilitation."

(1) When a nerve is severed, or when its trunk is partially injured or compressed, the impulses traversing one fiber can act to stimulate a neighboring fiber.<sup>3</sup> In some fashion, the injury robs the fibers of their normal insulation, so that an "artificial synapse" is established at the

site of the lesion. This interaction between fibers occurs most readily immediately after the injury is inflicted. The cross-firing may take place from motor fibers to sensory, or *vice versa*, although the sensory fibers apparently are far more susceptible to such aberrant stimulation than are the motor fibers; and of the various kinds of sensory fibers, the "C" fiber is the most susceptible of all. This is the small, "unmyelinated" fiber known to conduct the slow-traveling impulses which subserve a most unpleasant and persistent sensation of pain.

These laboratory experiments, based on careful electrical tests, demonstrate at least one way in which local lesion of a nerve trunk might become a direct source of sensory impulses.

(2) The second contribution from the experimental field which can be adapted to the hypothesis, is the concept of the "internuncial pool." This is the pool of neurons within the spinal cord, interposed between the posterior horn and the motor pools of neurons in the anterior and lateral horns. Within the internuncial pool, the incoming volleys of impulses are spatially and temporally dispersed before they activate the motor neurons. In addition to the dispersion of impulses, the internuncial pool acts as a switching and coordinating center which determines the routing of impulses to and from all parts of the central nervous system. In describing the activities of the internuncial pool, Gasser<sup>4</sup> has written, "A given stream of afferent impulses over a peripheral nerve follows one pathway in the centers at one time and another pathway at another time. The direction of the switching is conditioned by the situation obtaining at the moment, and is always consonant with a coordinated reaction of the whole organism"; and, "Anatomical peculiarities of the form and arrangement of the endings differentiate the ease of transmission spatially, and the nature of the previous activity differentiates it temporally"; and, again, "Ultimately excitation in a pool of neurons is dependent upon everything which is taking place in the nervous system anywhere because of the direct representation of this activity in the population of endings in the pool."

With this concept of the internuncial pool in mind, it is not difficult to imagine how impulses from a chronic source of irritation might act to disturb its switching function and its perfect synchronization of motor activities.

(3) The third laboratory contribution which seems to fit in with the hypothesis is the experimental demonstration of the phenomenon of facilitation. There are many examples of facilitation, but I have selected for presentation one that has been suggested as offering a possible clue to the hyperesthesia which occurs in causalgia. In this experiment,<sup>5</sup> rhythmic stimuli from an induction coil are applied to the posterior tibial nerve of a spinal cat. The strength of the stimulus is adjusted, until it is just sufficient to produce a reflex contraction of the *tibialis anticus* muscle. The minimal contractions of this muscle are recorded on a slow-moving drum. The phenomenon of facilitation is now demon-

strated by applying a variety of mild cutaneous stimuli to distant parts of the cat's leg. For instance, rubbing the side of the cat's foot with a wooden applicator, or blowing air strongly across the fur of the foot, will cause an immediate and marked increase in the magnitude of the contractions taking place in the *tibialis anticus*. That these strong contractions cannot be directly ascribed to the cutaneous stimulation alone, can be shown readily by stopping the induction coil shocks, whereupon the muscle contractions promptly cease. With the resumption of the rhythmic shocks, the facilitation of the flexion reflex continues as long as the light cutaneous stimulation is maintained. As soon as it is stopped, the reflex contractions of the muscle return to their former minimal type of response.

This experimental demonstration of facilitation in the spinal cat is interpreted by the authors as suggesting that "such facilitation may have a relation to the production of paroxysmal pain from light cutaneous stimulation in patients suffering from pain following nerve injury (causalgia)."

Critical examination of the three laboratory contributions just presented will reveal that none of them completely answers any of the questions propounded earlier in this paper. The first experiment, to demonstrate the cross-firing of fibers at the site of a nerve lesion, furnishes one possible source of the sensory impulses which we have postulated as being the original source of the vicious circle. It fails, however, to explain why so few nerve casualties develop the syndrome of nerve irritation, or why, when it does develop, its manifestations can be so excessive. The concept of the internuncial pool is of great value in permitting us to visualize the agency by means of which normal integrations are achieved between different parts of the nervous system. Yet the experiments which have demonstrated this agency have been performed on normal animals, and never have been carried out in human cases suffering from causalgia. To amplify the neurophysiologist's concept, as I have done, may be totally unwarranted. I visualize the activities of the pool as changed into a "whirlpool," to put it that way, fed by incoming impulses until it acquires a self-sustaining momentum, drawing into its powerful vortex more and more distant neuron systems, and becoming increasingly difficult to control by any therapeutic method. Though the phenomenon of facilitation fits well into our hypothesis, it cannot be used to account fully for the causalgic state. The experiment I have cited might be used to explain the local hyperesthesia of the affected limb in a case of causalgia; yet it could not account for the hyperpathia of the skin of the entire body.

Much experimental work will have to be done before we can furnish a clear answer to any of the questions propounded. Yet the experimental evidence already available definitely furnishes elementary tools with which we can expand and give support to the hypothetical formulation



which has been suggested to account for clinical observations. When we have solved the riddle of causalgia, I feel sure that we shall have gained an important insight into all forms of nerve irritation. We can then expect to attain better control over human suffering.

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### Discussion of the Paper

DR. GERHARDT VON BONIN (*Department of Anatomy, University of Illinois College of Medicine, Chicago, Illinois*):

The fact that relatively minor injuries to trunks of peripheral nerves cause intense pain requires explanation. One might assume that, normally, the epicritic fibers which are more heavily myelinated have an inhibitory influence on the gelatinous substance of Rolando in the posterior column of the spinal cord. The fibers of the medial moiety of the posterior root send collaterals in a sweeping curve from the posterior funiculus, through the *nucleus proprius* to the gelatinous substance, entering it, figuratively speaking, through the back door. They may well have, upon conduction, an electrotonic effect opposite to that from the lateral moiety of the posterior root, entering the gelatinous substance from "in front." Head's original observation that protopathic sensations alone are of greater intensity than when both epicritic and protopathic sensations are present, would support this view.

The mechanism by which causalgia can be broken remains, of course, completely in the dark. It is obviously similar to that underlying the success in shock treatment of neuroses or psychoses.

DR. MARGARET MEAD (*The American Museum of Natural History, New York, N. Y.*):

In connection with this paper, it may be important to recognize the similarities between menstrual pain and the type of diffuse heightened irritability to visual and auditory stimuli, and the behavior of the causalgia patients whom Dr. Livingston has described. It is possible that these recurrent hyperesthetic states in dysmenorrhea may be dependent on some chronically reactivated reverberating system of a related sort.

# A RECAPITULATION OF THE THEORY, WITH A FORECAST OF SEVERAL EXTENSIONS

By WARREN S. McCULLOCH

*Department of Psychiatry,  
University of Illinois College of Medicine, Chicago, Illinois*

IT is important to recapitulate, at this time, certain aspects of the theory of teleological mechanisms, and to indicate one or two directions in which progress is to be expected.

The participants of this conference have come together from such unlike disciplines of science that they have had to learn each other's vocabularies before they could hope to understand one another. This has made it difficult to follow arguments which in themselves were often relatively simple. I shall try to rephrase these arguments in ordinary language, although I am sure that, in so doing, I shall often fall far short of exact or rigorous statement. To make up for this, I shall try to relate the novel notions to the history of thought, so that it can be seen how we came by them.

The conference has considered two related questions; namely, "What characteristics of a machine account for its having a *Telos*, or end, or goal?" and "What characteristics of a machine define the end, or goal, or *Telos*?" It has answered the former in terms of activity in closed circuits, and the latter in terms of entities which the mechanism could compute in terms of the discriminations it could make. We are all indebted to Thales for the basic conception. He was the first to insist that the Gods were in things and not behind them. To know the Gods, then, is to know how things work. Unfortunately for physics, the Greeks thought that generation rather than motion was the cause of apparent change. Generation determined what it was, and what it was determined what was the natural place for it to go. For other than living things, this place, or *Telos*, lay outside of it, whereas for living things the *Telos* lay within it. *Entelos*, the end of the operation, was within the operation. Fortunately for biology, the basic notion of function was, and remained, an operation whose end was within the operation. When the notion was imported into mathematics, it had the same meaning: *Y* being a certain function of *X* meant that *Y* was the end inherent in the given mathematical operation upon *X*.

Aristotle, however, used the end in operation to obtain a law of the conservation of species, as we use potential energy to obtain a law of the conservation of energy. Neither entelechy nor potential energy is supposed to *do* anything. The future does not operate to alter the present. Only the ergs within it, the energies, do that. But the kind of bird which the egg will become is the end in, and of, the operation within, and of, the egg. It shall try to incorporate only such material as shall be suitable

to that end. Everything else is evil for it. Everything suitable to that end is good.

But living things do not live by themselves. Men may exchange some of one kind of thing for some of another. To facilitate this, they introduced money as a common measure of value for marketable goods. The question then arose whether there was, conceivably, a common measure for all values. From this developed, eventually, the normative sciences of ethics, esthetics, and philosophy, including, in the last, logic and mathematics. So teleology stood with the good, the beautiful, and the true when Roman engineering overwhelmed Greek science, and was lost in the Dark Ages. Then Thomas Aquinas picked it up where Aristotle had left it and made of it the sublime *Causa Finalis* which extended as a veritable hierarchy of values from lowly creatures to the God who stood apart from or behind them. Ironically, it was this separation of the Divine Cause or *Telos* from the natural cause or *vis a tergo* that permitted good Christians to investigate the laws of physics.

Centuries passed, though, before the first teleological machine was made by Watts, who constructed a governor for a steam engine in 1784, and nearly 50 years more before Sir Charles Bell published the first closed path in an organism (*The Nervous Circle*, February 16, 1826). By 1850, the French school of neurophysiology had defined the reflex as an activity which, originated by a change in some part of the body, proceeded to the central nervous system over the dorsal roots, whence it was reflected over the ventral roots to that part of the body where it had been initiated, and there diminished, stopped, or reversed the change that had given rise to it.

This is exactly what the governor on a steam engine does. Although Maxwell published the mathematical theory of its behavior in 1868, and Wischnegradsky in 1877, the trick had to be independently rediscovered in many arts. I can remember the old bimetallic strips we used in thermal regulators, the introduction of sensitive electromagnetic relays, and the first use of a vacuum-tube relay to obtain better thermostasis. That must have been about 1920. By 1930, self-regulating power supplies were being built into well-designed vacuum-tube devices, although most electronic engineers may date the introduction of inverse feedback amplifiers some four years later, when Black published his work on telephonic repeaters.

In the laboratory, factory, and home, in any machine, we could regulate anything if we could devise a sufficiently sensitive receptor and appropriate effectors to control the variable desired to hold constant. We could make the value of that variable to which the system returns follow any course we desired.

Cannon showed how living organisms likewise hold constant many crucial variables. This he called homeostasis. He even pointed out how the value of the variable to be sought by the system might be altered by

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demands made upon it, and finally he extended the notion to the relation of the system to the environment.

It had become clear that the problem, whether in organisms or man-made machines, was a question of signals in closed paths. Considerations of energy were immaterial. It was information that went around the circuit. Then began the collaboration between the physiologist, Rosenblueth, who was Cannon's chief collaborator, and the communication engineer—in this case Norbert Wiener—a mathematician if you will. In January, 1943, they published with Bigelow, in the Journal "*Philosophy of Science*," an article on *Behavior, Purpose, and Teleology*, which heralded such discussions as we hear these days. They concluded that purposeful, or teleological, behavior is controlled by negative feedback.

Let me resume what all of the systems have in common. Each has a path which is closed. Each has a natural period, that is, the time necessary for the signal to complete the circuit. Each has a gain, that is, the ratio of the size of the initial disturbance to the size of the disturbance returning to its origin. The gain, generally, is different for different frequencies and for different sizes of disturbances. The feedback is regulatory only when it opposes an induced change and the gain is less than one. If it is equal to one for a given frequency, the size of the disturbance oscillates. If the gain is greater, oscillations increase until the system is destroyed or, at the greater size, the gain becomes one when it continues to oscillate at that size. The intrinsic disease of negative feedbacks is, one and all, that they cease to be negative. This state may be enforced by compelling them to operate at other frequencies than those for which they were proportioned, or may be permitted by altering the natural period or increasing the amplification of the signal. As soon as feedbacks operate with a gain greater than one, they cease to subserve their proper ends.

There are, in organisms, many processes which are rhythmic and of remarkably constant amplitude. Some of them are sinusoidal rhythms, others decidedly not. We can divide their circuits into two groups. In the first, if we *add* causes, we *add* effects. We call this group linear circuits. If they oscillate, the oscillation is sinusoidal, and only sinusoidal. In the second group, adding causes does not simply add effects. We call this group non-linear circuits. If they oscillate, the oscillation will have a form which may be far from sinusoidal. To this, the non-linear group, belong the so-called relaxation oscillators. Both kinds of circuit can be found in the body. However, most circuits are linear only for relatively small excitations. They are usually so constructed that, as the amplitude increases, the gain decreases, affecting chiefly the extremes of the deviations from the central position in one direction or in both. At this point, they have ceased to be linear. Hence, the general case is the non-linear one. However, the mathematical description is simple only for the linear case. Hence, for the non-linear case, wherever possible, we will juggle

the mathematics so as to find a new variable, say the logarithm of the original variables, in terms of which the oscillation is sinusoidal. Moreover, in the body, some circuits are easily identified through their entire closed path; others are diffuse, both here and there; and many have a variable portion of the path, now here, now there.

But whether they are linear or not, and whether they are well defined in path or not, in these circuits cause and effect are so closely interconnected that it makes little sense to ask whether the bird or the egg was first. Each event bears such an imprint of its precursor that its consequent resembles that precursor. And that was all that Aristotle needed for his conservation of species, the active repetition of the same form. Thanks to Lorente de Nó, we are certain that, in the nervous system, activity does persist in closed circuits of neurons, is responsible for nystagmus persisting long after stimulation has ceased, and may account for a very active memory in which the occurrence of a certain event may be retained without reference to the instant of occurrence.

We may divide all inverse or negative feedback devices into those which are merely homeostatic, *i.e.*, keep some internal parameters of the system constant; those which are "servo," *i.e.*, those in which the value of the parameter sought by the system can be altered from without the circuit; and the appetitive, *i.e.*, those in which the circuit passes through regions external to the system. But such distinctions are a bit arbitrary.

We have seen that the end sought by the system may be any parameters we choose, or to which its receptors happen to be sensitive, and hence that the ends sought by such systems may have no common measure, or be dimensionally dissimilar.

Consider an organism in which two such systems, of which it has many, are active at the same time and would require opposite actions of one or more parts of the organism. Working concurrently, they would destroy it, as, for example, swallowing and inhaling. There must exist some connection between the circuits, so that in case of conflict one inhibits the other. The same must hold whenever the world makes us choose one of two ends. One circuit must dominate or we die.

In many cases, we are like a man driving toward a cliff. We may turn right or left: which way, is indifferent, only we must turn. We have the ability to decide or we should not have come to attend the conference. Even when there are two things which we must do to survive, we must choose, and there is often more urgency to the one than to the other. We must do the more urgent immediately and the other when we can. This other, consequently, remains active in us and unfulfilled. Thus it comes about that many a natural but long-term need lives on until the morrow, when with accumulated urgency it demands fulfilment. Whether or not we are aware of them, we are all freighted with such active processes, which at times erupt inopportunistly. These are diseases not of a single negative feedback, but of the interaction of such systems.

Apart from urgency and the conditions of the moment, values do

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not constitute a hierarchy. By a series of tests with rats, it can be determined at what degree of starvation for both food and sex half of the experimental animals will prefer the one and half the other. Economists have called the plots of these values indifference curves. We could also plot how much punishment would prevent half the rats from seeking food, or half the rats from sexual activity. We should now have these indifference curves, but a strange thing appears. If we assume that values have a common measure, then rats starved for both food and sex so that they choose half and half, ought to be willing to take the same amount of punishment to get either. But this is not the case. They will take far more punishment for sex than for food. In fact, rats may be starved so that they will almost all prefer food to sex, and yet in that condition they will prefer sex to avoidance of pain and avoidance of pain to food. We have, under these conditions, three teleological mechanisms so interconnected that the first dominates the second, the second dominates the third, but the third again dominates the first. I ran into a similar circularity of preference more than 20 years ago in experimental esthetics, but could make nothing of it at the time. Unfortunately for the theory of motivation in psychology and economy and the hierarchy of *Causae Finales*, values are not magnitudes of any one kind any more than ends are dimensionally similar. Both differ in kind, not merely in degree, and we must look to the mechanism for the appropriate theory. Therefore, as to the good in the biological sense, and the beautiful in the sense in which it can be inferred from mere human preference, we indeed live in a world of many incomparable values, where fate compels us to choose whenever we act and nature does not forgive us for leaving undone those things which we ought to have done just because, then and there, we had to do something else.

I should commit precisely that error if I did not now mention what has been one aim of this conference, namely, the extension of the realm to which these notions are applicable. Organisms do not live by themselves and may be links in closed paths through which the feedback is negative. We have heard examples presented from Ecology, Cultural Anthropology,\* and Public Opinion.\* There seems to be little question but that feedback occurs in those fields and is not always negative.

In the discussion of the examples, predictive problems came to the forefront. During the war, as we know, this theory was advancing. It had been incorporated in certain engines of death and destruction which enabled guns to shoot at the place where the target would be when the shell arrived. This they did by autocorrelation of data in time for a variable lag. There is a story that Leonardo da Vinci killed the mechanic who worked with him, lest knowledge of the submarine reach the ears of some would-be conqueror. I cannot imagine that Norbert Wiener feels happier about the way the theory of prediction is being used, but it was he who first demonstrated that there was an optimal prediction and how

\* Unpublished papers presented at the Conference.

to achieve it. He has pointed out how such autocorrelations and inter-correlations of data in time-series may detect the existence of causal connections, and their lags in one or both directions, provided the correlation is less than perfect. He has also demonstrated how, by these means, we may detect feedback, including inverse feedback, in Ecology, Anthropology, and Sociology. The data need be no more than decisions, actions or opinions in time, provided we have runs of sufficient length. I know of no utopian dream that would be nearer to everybody's wishes, including my own, than that man should learn to construct for the whole world a society with sufficient inverse feedback to prevent another and perhaps last holocaust. There may at least be time for us to learn to recognize and decrease the gain in those reverberating circuits that build up to open aggression. We cannot begin too soon. To make it quite clear, we have no desire to reinfect a convalescent sociology with the virus of that attempt to better the world which is proper to medicine and engineering, which are not sciences but arts. All we have a right to ask of the appropriate sciences are long-time runs of data. We know it will take years to collect these, but we must have them before we can determine whether the mechanism of negative feedback accounts for the stability and purposive aspects of the behavior of groups. This was one of our questions.

The other end concerns the discriminations of which the machine is capable, and what it can compute in terms of them. Because Democritus took seriously the riddles of Zeno, he gave us the notion of a least, or atom—something which was there or else was not there, and that was all there was to it. It had for him no other properties. Our chemistry was born with the notion that atoms differed in kind and could be combined to form molecules, which had properties that could not be deduced from the addition of similar properties of their component atoms. Fortunately, we are in a slightly better position with respect to the activity of the nervous system. We know that each action of a nervous cell is atomic: it either happens or does not happen at a given time. We can regard each cell as a telegraphic repeater which, on receipt of an appropriate signal or combination of signals, emits one of its own. It has a threshold. After a brief impulse, there is a short period in which received signals may add to exceed the threshold of the cell or remain to prevent its firing, then a somewhat longer delay before it emits its signal, and a roughly equal interval during which it is unexcitable after activity. Most of these properties of the nervous impulse have been accurately measured in New York by Lorente de Nó, and its inhibitions demonstrated by David Lloyd. Let us consider any cell, *C*, as receiving either a single adequate impulse or an adequate impulse and, at the same time, an inhibitory one which may prevent it sending on an impulse. Let us consider these impulses as coming from two sources and call them *A* and *B*. Now since *A* and *B* are atomic, there are only two possibilities with respect to each: the impulse either happens or does not happen.

There are, then, four cases conceivable: only *A*, only *B*, both, or neither happening. The last of these could never excite *C*. There are, then, eight ways in which the excitation of *C* might be related to the occurrences *A* and *B*, one of which is the trivial case, inconsequentiality, in which *C* does not get excited at all. The signal *C* may represent either *A*, whether or not *B* occurred; *B*, whether or not *A*; both; either *A* and not *B*, or *B* and not *A*; or, finally, *A* or *B* or both. These are the functions out of which one can construct the complete calculus of signals. Given memory and enough cells in proper circuits, such a nervous system can detect any combination of afferent stimuli in time and space. It can convert any pattern in space into one in time and *vice versa*. It can compute any computable number, and, given the time required, arrive at any deduction from a finite number of premises.

An ordinary brain, a merely human brain, has only about 10 billion nervous cells, that is about  $2^{23}$ , whereas the eye alone has more than a hundred million photoreceptors, each of which is either signaling or not signaling at a given moment. This means that the eye can exist in  $2^{100,000,000}$  states, each of which corresponds to a unique distribution of stimulation. It is obvious, therefore, that man's brain, even if it had no other function, could not have a single cell to respond to each distribution even if it could contain all the necessary connections, which it cannot. Nor would it be of any use to man to bring all this information into his cortex, spread over an equal number of elements. What he does in the three layers of the retina is to note masked coincidences and abrupt changes, and send in this abstract of the data over about a million fibers from each eye to the lateral geniculate ganglia. There, coincidences and displacements in the two eyes are, perhaps, noted. In any case, the information is sent on in three ways: to the cortex for detailed analysis; to the superior colliculus to help determine the proper movement of the eye to keep it turned to what is important; and to the great master servomechanism of motor control, the cerebellum. In the primary optical cortex, contrasts are heightened, and incomplete data filled in and thence relayed to the secondary cortex where stimulation at a point gives perception of formed objects. At the very step when perception of form occurs, the last vestige of conformal representation disappears. This is only five relays removed from the stimulus, yet a lesion here may cause imperception of hue with perfect ability to match colors, and, a few millimeters away, alexia. Perception of hue may, or may not, be learned. Reading is learned, yet it clearly depends on local structure, and that local structure must depend on use. Nervous cells are alive and presumably grow with use. This is exactly what Ramón y Cajal proposed. Lorente de Nó would prefer the hypothesis that they and their connections diminish with disuse.

We may picture the process somewhat as follows: Imagine a nervous cell so connected to a second cell that it can excite it, and then imagine a third cell which is not quite able to excite the second cell. Let our law



of growth be such that if this third cell is excited while the first cell is firing the second, then the third cell will so grow as to be able to excite the second, or, what amounts to the same thing, let the second cell so grow as to become excitable by the third. This would give us the fundamental kind of change in structure produced by use, in terms of which to account for the conditioning of a reflex. Excitation of the first cell is effected by the so-called unconditioned stimulus, the second cell being the path to the response, and excitation of the third cell by the conditioned stimulus. Such an altered structure yields a passive memory. The introduction of such a passive memory into a net which contains no closed paths leaves its calculus that of signals. In this, it differs from the introduction of a *tertium quid* which might be a diffuse background of excitation, a neuron undergoing relaxation-oscillations, or a regenerative cycle of activity in a reverberating chain of neurons set going by a stimulus in the remote past. Introduction of any of these makes it possible for the system to respond to mere absence of excitation at some specific point, and thereby creates the possibility of including response to that case in which neither of a pair of atomic events occurs. This alters the appropriate calculus from that of signals to that of signs. This calculus has now sixteen, instead of eight, functions of two arguments, and in this respect it becomes equivalent to the full calculus of propositions, from which it differs, however, in that one still has to keep track of the time of occurrence of the proposition, although no longer of the event proposed. When passive memory is included in such a system, or, what amounts to the same thing, when the organism has effectors with which it can make enduring marks or signs, and sense organs with which it can resense them at any desired time, it can neglect the time of occurrence and think in terms of the calculus of propositions. The use of such signs is, in fact, equivalent to the incorporation into its nervous system of an infinite number of reverberating circuits running for all the required durations. Moreover, by means of these signs, one organism may share any task of computation with another similarly conditioned. Thus, it comes about that the world of metamathematics, as well as the physical world, is public property.

But let us return to the actual brain with its paltry ten-to-the-tenth neurons. It seems fairly clear that the genes which inhabit our tiny chromosomes cannot contain a number of particles sufficiently large for their order and arrangement to specify in detail which nervous cell is to be connected with which. They can, in fact, at best serve only as a pattern for the more general features of order and arrangement, leaving to chance and learning the detailed connections, or fine structure, of the net. This is neither the time nor the place to go into the mathematical theory required for a statistical approach to this structure. It is sufficient, for the moment, to state that this can be approached by Wiener's method of auto- and intercorrelation of the activities of many points in the net. What was required was the development of a theory in which

the value of the relations of activity of neurons could be approximated by the first term of a series in ascending powers. This has been achieved by Walter Pitts, by selecting, as a value to satisfy this relation, the chance of the firing of a particular nervous cell that is equal to the chance of afferent impulses being transmitted to it from other neurons in the structure. The empirical procedure for determining the statistical properties of the structure is, then, to administer to one point in an isolated portion of the structure a series of equal electrical pips of random distribution in time, to record their arrival as propagated disturbances at several remote points in the region, and to study their auto- and intercorrelations as series in time.

All one can hope to obtain from such measurements is this: Structures may be chaotic in an infinite number of ways, and these statistical measures, even though they exclude an infinite number of possible varieties of chaos, will still leave us with an infinite number of possible varieties. The exclusion will rest on our knowledge of the distributions in time and space of the transmitted pips, from which we can conclude something about the number of junctions between two points and the frequency of occurrence of closed paths having two, three, four, five, or more junctions within them. It is certainly to these little circles that we must look for the most transitory forms of active memory. We propose to start work on the bark of the brain, first perhaps on the visual cortex, beginning with the primary optic area, and then with the secondary optic areas where each position stimulated yields perception. After that, we shall proceed to typical associative areas. This should give us a more useful working knowledge of the properties characterizing the physiological performance of the anatomic substrate of psychology.

The question we should like to consider at the moment is how it comes about that we perceive any form whatsoever. We already know that we have not enough nervous cells to have one respond to each of the distributions of activity of which the eye is capable at any instant, nor enough fibers to relay the information from the eye to the brain. We know that in the eye itself the cells are so connected as to respond to coincidence of activities, or to coincidence except for a small fixed time-delay. It is information of this sort which is transmitted over the optic stalk. Thus, whatever perceptions the brain is to form must be built by recombinations of these abstractions. Let me contrast this with any supposition that there is a conformal pattern of stress and strain in the brain, comparable to that which might be found in a system in equilibrium when the forces of the external world determine that pattern. First, the brain is a system which is not in equilibrium, either within itself or with the outside world; and second, the pattern of the stimulation from the outside world is partially abstracted even in the retina. At the geniculate bodies, it may again be coincidences, this time from the two eyes, that count. The information passed on by them to the cortex, concerning the dimension of depth, is in no sense conformal with three-dimensional

space or its depth, but is simply a matter of impulses passing at specified times along linear conductors. However, this much is true, that some of this information is reassembled in the primary visual cortex, which has a point-to-point correspondence to the retina and thus to the visual field, although it is not strictly conformal, since it is divided in two in the mid-line and, at this line of cleavage through the point of foveal vision, abuts the secondary visual cortex. Now this latter cortex has recently been shown, under conditions of hyperexcitability, to exhibit a point-to-point relation of activity to retinal stimulation. As yet, we do not know the anatomy of the path; but, be that what it may, the point-to-point correspondence here is the reverse of that in the primary visual cortex, and local stimulation of the secondary visual cortex in man yields perceived form instead of local bright spots which follow similar stimulation of the primary cortex. During the last year, a certain amount of information has begun to point toward the representation of seen motion in area 19, which is still further removed from the primary visual cortex. Also, in looking back over old work on electrical stimulation of the cortex, it is stimulation of area 19, or regions anterior to it, which has yielded perception of moving forms and dizziness in patients on the operating table. A second consideration might lead us to the same conclusion, namely, that motion is represented elsewhere than form in the cortex. When a man is tired, his eyes, instead of snapping from one position to another, move slowly, and he becomes unable to keep the forms from blurring. Apparently the perception of form requires appreciable time for the coincidence of impulses over paths of unequal numbers of relays. This is presumably the chief reason for the existence of the many and complicated servomechanisms that keep the eyes fixed on objects in space, however we or they chance to move. Also, it is apparently the function of the primary visual area to determine the form as precisely as possible, which means that it is not suited for handling motion. For years, introspectionists have noted that it is easy to recall the visual image of a familiar room as it appears from almost any point they have ever occupied, but that it is extremely difficult or impossible to let that point move in the room as if they were walking into it. The visual image is retained only as a series of clearly distinct snapshots. From this, it seems likely that area 17 and, perhaps, area 18—the primary and secondary visual areas—are those whose structures retain, permanently and passively, some impression from visual experience of form, and that it is these structures, reactivated by impulses from other parts of the brain, which yield the visual image devoid of motion.

I have spoken of the visual system, although I might have spoken of any other sensory system equally well, and I have labored the point because so much of the work in Gestalt psychology has been done in the field of vision. The observations made have been invaluable, but, as the founders of the school foresaw, their theories have encountered almost insurmountable obstacles because of our lack of knowledge of nervous

processes. From adequately developed neurophysiology, we should be able to deduce most of the relations characterizing Gestalt psychology. This much, at least, should be obvious, that psychology is fundamentally sound in assuming that perceived forms cannot be reached by a simple addition of their component parts. The nervous system never works that way, as we have seen in the case of clonus, wherein, because nervous cells respond to coincidence of disturbances, the response may be sinusoidal in the logarithm: that is to say, it is a product, not a sum, that counts. Now the body of man—his effectors—has an enormous number of degrees of freedom, but they are by no means as numerous as the forms of excitation of the retina, nor can they be executed with anything like the speed at which the retina can change in a changing world. Perfect conformal representation, were it to go all the way through the system, would jam the effectors. We have more information than we could possibly use even if our brains were big enough to handle it. Hence, the brain must abstract a smaller set of patterns for behavior. Even if it could and did abstract more, we could never know this about someone else, for he could never communicate it by behavior.

At this point, let us turn back to our initially random net and ask how it ever comes about that, in such a net, order is introduced so that there are objects in the world for us. It will doubtless be recalled how the associational psychology of Aristotle was simplified by the British school into association on the basis of likeness and association on the basis of togetherness in time and space. It is clear that we cannot use association on the basis of likeness to explain the origin of semblance itself. This was painfully clear to Mill, who suggested, as a way out of the difficulty, that those things are initially alike for us which have been associated in time and space throughout the development of the kind. In other words, let us suppose that we inherit some structure, as we do, which, instead of being altogether random, has laid down initially the paths required for detecting some similarities. The counterpart of this psychology is Kappers's law of neurobiotaxis, namely, that phylogenetically those cells which are associated in activity become associated in structure. The law is at least descriptively correct in very many instances, and there is nothing in it, or in Mill's suggestion, as to the mechanism underlying phylogenesis. It is neither Lamarckian nor Darwinian, merely factual. But it states this much, that historically with evolution of nervous structure, new semblances have become recognizable (and, harking back to Aristotle, we may add, new differences discernible). It is clear that the same thing happens in the introduction of new ideas or the recognition of new forms within the span of a single life, though obviously the mechanism cannot be the same.

Now let us turn back to Cajal's theory of learning and suppose that, in the growing nervous system, cells used together become associated in structure. In our initially somewhat chaotic nervous system, almost everything might be connected to almost everything else. Clearly, there

must be competition between nervous cells for footspace on their followers. Then, at least, priority in time might determine which would succeed and be perpetuated, but it is to be feared that this is not enough. Confronted with a new problem, behavior may be initially random, but once success is achieved, the successful mode of behavior becomes the preferred mode, and ultimately the fixed mode, of behavior. This, in substance, is Thorndyke's law of effect. The question is simply how this can be accounted for in terms of what will fix the connections of cells. Must we invoke impulses from some remote structure whose activity corresponds to its sense of satisfaction, or can we look locally to the relations of one cell to another? I believe the latter is the case, although as yet I see no way to prove it. We will invoke what may be called a setting-in process. It will be recalled that, if an external magnetic field is applied to a lump of iron, the little magnets scattered within it are compelled to assume new positions and, if they are subjected to a series of such forces, the strength and duration of these applied forces will, to a certain extent, determine their organization. The most significant thing by far will, however, be that force which last put a given magnet into position before the force disappeared. Our setting-in process is comparable to this. The activity itself disappears when the problem is solved, and it leaves the cells to continued growing along the last pattern enforced by the activity. This, too, then, is but another example of the importance of inverse feedback. The activity of these self-same cells, relayed through effector mechanisms, has brought their own activity to an end and left us with a new idea, embodied in a new structure.

Let me point out that the foregoing rests on two hypotheses: that use determines growth and that rest determines set. Now, all lay opinion to the contrary notwithstanding, every scientist knows that no hypothesis can ever be proved. It can only be disproved. Suppose I give the numbers one, two, three, and ask for the next number in this series. I have the hypothesis that the answer will be the next integer, four, but it might equally well be "five." This would disprove the hypothesis of integers and I might form the hypothesis of the primes and ask for the next number, expecting seven. This hypothesis would be disproved if the answer were "eight." My new hypothesis might be the Fibonacci, so that I would then expect "thirteen" and any other number would force me to a new hypothesis. But every mathematician knows that no finite number of numbers determines a unique rule for the formation of a series. There are, in fact, an infinite number of rules that would yield the same finite number of numbers in the same order. For any given number of numbers, there are, then, an infinite number of hypotheses which are equally tenable and an infinite number which are false. It would require an infinite number of numbers, in other words all the numbers of the series, and the knowledge that such was their total, to define the rule of formation. This alone could prove the hypothesis true, and then it would be no longer necessary or useful in the construction of

the next. A somewhat similar phenomenon occurs when we attempt to measure the wavelength of light or the pitch of sound. It cannot be, ultimately, precisely defined unless it lasts forever.

As Prescott has shown, quite apart from this difficulty, through the perturbation of any parameter we would measure by the effects of variables beyond our control, and unnoted in our equations, the form of every function in physics either lacks empirical foundation or remains ultimately undefined. We are, after all, finite creatures, and there will always remain for us an infinite number of formulations of experience which are equally true or else false; many that are false; but none of any importance for the morrow which is indubitably true. Science, unlike the hungry man who may find food, cannot hope for an inverse feedback that would supply a satisfactory, all-inclusive system of true propositions as to how the world works.

### *General Discussion*

DR. F. H. PIKE (*Columbia University, New York, N. Y.*):

Changing the symbol of one variable in a formula previously published,<sup>1</sup> we may represent the neuromuscular portion of respiration as

$$R=f(P) (V) (T) (N),$$

in which respiratory activity  $R$  is expressed as some function  $f$  of the pressure ( $P$ ) of certain gaseous substances in the circulating fluids, the volume ( $V$ ) of the fluid passing through the central nervous respiratory mechanism in unit time, the temperature ( $T$ ) of this fluid, and the nervous factors ( $N$ ) necessary for respiratory movements. Increasing the pressure of  $\text{CO}_2$  in the fluid in the central nervous mechanism, or reducing the volume of fluid passing through it in unit time, increases the pulmonary ventilation. Increasing the temperature of this fluid may give either an increase in total pulmonary ventilation, an increase in the volume in unit time, as indicated by a rise in arterial pressure, or some combination of both. The general response of the central mechanism and, through it, of the organism, is interpretable (*a*) in terms of the theorem of le Chatelier,<sup>2</sup> in that the system tends to respond to any constraint imposed upon it by a change in any parameter, or parameters, by a change, opposite in direction, tending to neutralize the constraint so imposed; and (*b*) in terms of the law of mass action,<sup>3</sup> in the sense that constancy of concentration tends toward maintaining a constant velocity of reactions. ( $P$ ), ( $V$ ) and ( $T$ ) have the same significance they have in the gas laws, but the quantitative determinations in the organism cannot be brought to a degree of accuracy that would enable us to compute any one variable when the other two are given, as can be done in isolated systems of gases. In general, it may be said that the changes in the volume of fluid flowing through the central mechanism are in such a direction as to maintain a constant pressure of the gaseous substances. The

biologist will recognize that pressure, temperature, and volume are important parameters in that group of factors in the environment which he calls the conditions of existence.

The nervous factor (*N*) has been held by some to be independent of any inflow of afferent impulses, *i.e.*, to be automatic in its reactions. I must confess that I fail to see the compelling force of the arguments urged in support of this view. It is true that the central respiratory mechanism will discharge when it is in such a state that afferent impulses sent in over the vagus or the sciatic nerves<sup>4</sup> do not change its rate or character in any way, but an animal in such a condition will not live without artificial respiration continued until well beyond the stage at which afferent impulses again become effective. I do not know of any other experimental proof in which afferent impulses can certainly be excluded. Certainly, no animal higher than a frog in the systematic scheme will live when all the afferent impulses flowing into the central mechanism, or even the greater part of them, are eliminated. In the frog and lower forms generally, it is not possible to eliminate, experimentally, all the afferent impulses without direct involvement of any efferent nerves. Alligators promptly die when the dorsal roots of the thoracic nerves are cut, without touching the vagus (unpublished results).

It is necessary to consider this question of the automaticity of the respiratory movements in connection with the hypothesis of inhibition of respiration. It is difficult to see how, if the central respiratory mechanism is purely automatic in its action, any minor disturbance of the afferent impulses coming into the central mechanism, such as that related to an act of swallowing, could have much effect upon it, or, differently stated, how respiratory movements could be inhibited during any part of the act of swallowing. One may grant, as indeed one must, that there is an independent irritability of the central respiratory mechanism, but this does not mean either that respiration is automatic, or that inhibition is a valid assumption. Independent irritability of the central mechanism is just as necessary as a condition for the hypothesis that respiratory activity is dependent, in part at least, upon afferent impulses from the respiratory muscles, as it is for the hypothesis of automaticity. If the respiratory mechanism is to be brought within the scope of the central theme of this conference as a feedback mechanism, the necessity of afferent impulses from the peripheral respiratory motor mechanism must be shown to be probable. The hypothesis of automaticity does not necessitate all the phenomena<sup>5</sup> and leaves much unexplained, whereas the hypothesis of afferent participation does necessitate all the phenomena.

The muscular apparatus of the respiratory mechanism has commonly been regarded as only that which gives rise to the movements of breathing. However, few who have observed animals under experimental conditions can escape the conviction that the whole cardiovascular mechanism is also involved in any organism as complicated as, for instance, a

cat. It is to the cardiovascular mechanism that we must look for the changes in volume of blood flowing through the central respiratory mechanism in unit time. Without such changes, animals would be helpless in emergencies. When tyramin, which constricts peripheral blood vessels strongly, is injected, under brief anesthesia, into the *cisterna magna* of a cat, the animal lies panting on the floor, apparently unable to move about despite the rapidity of respiration (unpublished results). But, considering the respiratory mechanism as that mechanism in which the visible external muscular movements are taken as constituting the mechanism of respiration, this mechanism has undergone a profound change, perhaps more profound than any other, in vertebrate evolution. In sharks, all the important motor nerves are cranial nerves, and all the muscles are those concerned with movements of the mouth and gill arches.<sup>6</sup> Anatomical section of the dorsal roots of the spinal nerves does not affect the respiratory movements to any observable degree. Anatomical separation of afferent and efferent pathways is not possible, but there is good reason, anatomical and experimental, for supposing that the mesencephalic root of the fifth cranial nerve is a part of the afferent system. The whole tribe of amphibians either retains the motor and sensory functions of the cranial nerves in respiration, or dispenses with any motor mechanism except the cardiovascular to get oxygen and eliminate CO<sub>2</sub> through a wet patch of skin, beneath which there is a rich network of blood vessels. Costal respiration, or some modification of it involving spinal nerves, appears in reptiles, birds, and mammals, and no cranial nerve retains any important motor function in respiratory movements. The acquisition of a mechanism in which spinal nerves and somatic muscles took over respiratory movements was a major event in the development of land-dwelling vertebrates.<sup>7</sup> Johannes Gad's old statement that the nervous mechanism of respiration in mammals extends from the facial nerve to the lumbosacral plexus should be extended to include the fifth cranial.

However, the transition from aquatic to terrestrial habitat, accompanied as it is by a profound change in the peripheral nervous and muscular apparatus of respiration, has not been accompanied by an equally profound change in the central nerve nuclei of the respiratory mechanism. The principal central nucleus is located in the lower (caudal) portion of the *medulla oblongata* in fish and man alike, and the midbrain is as important in the cat as in the shark. In arriving at an estimate of the organization of the neuromuscular mechanism of respiration, we must keep in mind the primitive conditions in the shark (although one may suspect a somewhat different central mechanism in cyclostomes), whatever the type of animal with which we are dealing. I have presented what I consider to be the fundamentals of the organization of the central mechanism in mammals in an earlier paper.<sup>8</sup> I have seen no reason to change this scheme in any important particular since that time.

The hypothesis of inhibition does not seem applicable. Some of the



same muscles are concerned in the act of swallowing and in respiratory movements. Swallowing is an act which is elicited by excitation of certain nerve endings in the pharyngeal mucous membrane. It is a response to a particular excitation of a particular set of afferent nerve endings, and cannot be performed voluntarily except by voluntarily bringing something into contact with the pharyngeal mucosa. When the act of swallowing is completed, there is an expulsion of air from the lungs—the *Schluckatmung* of the German authors—no matter at what phase of the respiratory act the swallowing may have occurred. Swallowing and respiration are two different acts, each arising from a specific form of excitation, and the necessity for assuming that one must be inhibited in order that the other may occur is not immediately apparent. The snake does not need to inhibit respiration, in the sense in which inhibition commonly has been used or, at least, it is not immediately evident that it must do so, when it wraps itself about its prey to crush it, but respiratory movements apparently do not occur while the snake is crushing its prey.

Inhibition and facilitation are words of ancient, if not always honorable, lineage in the physiology of the nervous system, but the sense in which they have commonly been used, *i.e.*, to give a name to a process of unknown nature, leads one to suspect that frequently there lurks behind them what Emil DuBois-Reymond called the “thinly veiled specter of vitalism.” In dealing with mechanisms, as supposedly we are doing in this conference, we should look for terms which have a basis of some kind in mechanism.

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MR. DONALD HERR (*Control Instrument Company, Inc., Brooklyn, N. Y.\**):

While Dr. McCulloch's presentation reflects the neuropsychiatrist's research experience and viewpoint concerning circular processes, it may be useful to synthesize certain of his concepts with some which those of us engaged in servomechanism work are continuously forced to consider. The results should at least be stimulating, especially since, in servomechanism work, we are concerned only with systems whose totality of responses is utterly mechanistic and non-subjective, and this latter group seems to embrace all the response-attributes of the neural struc-

\* Present address: Allen-Bradley Company, Milwaukee, Wisconsin.

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tures to which Dr. McCulloch is restricting himself at the present time.

I believe Dr. McCulloch, in alluding to the aims of the organism, made the arresting statement that its desired quality is "stability, if only to avoid the opposite." The question can first be raised as to what one should consider to be "the opposite"—instability leading to the destruction of the organism, or instability leading to a change in the organism to a new, essentially stable condition? If the former is meant, then instability becomes synonymous with non-survival, death, or destruction of the organism (mechanism). If the latter, then instability *may* be an intermediate, transient condition potentially leading to a new condition of stable survival or operation.

The answer to this question and the attitude behind it seem to me to be of fundamental importance. Perhaps a few extrapolations from the engineer's experience in servomechanisms may help to guide workers in other fields in their attempts to utilize constructively the feedback concept. (See the illustration on page 192 of this monograph.)

We can confine ourselves to a discussion of a simple feedback system in which only one feedback loop exists between output (response) and input (stimulus), since more complicated systems, involving several or many complex feedback paths, are qualitatively subject to the same conclusions as those simpler ones. It is found that, when the nature and amount of the feedback are adjusted to give great stability, then the system becomes most sluggish and relatively inaccurate in its response. Conversely, when the nature and amount of the feedback are adjusted to give great accuracy and rapidity of response, then the system rapidly approaches a condition of instability of one kind or another. *Accuracy of response* and *stability of operation* appear as two absolutely interdependent, yet contrary attributes of the feedback system. In servomechanism work, we have to compromise degree of stability to satisfy the accuracy-of-response specifications, or accuracy-of-response to satisfy the stability specifications, depending upon the controlling requirements in a particular application.

In an organism, or in a neural structure, it seems that, qualitatively, the situation is entirely analogous. The controlling requirements in a particular application of a servomechanism correspond to the organism's environment (external stimuli and required responses). In one instance, great stability without too accurate a response may be necessary for the organism's survival; in another instance, great accuracy-of-response with a sacrifice of degree of stability may be required. In either case, either type of response may not be sufficient for the organism's survival, even assuming it have the ability automatically to adjust its feedback gain properties and, hence, its stability and accuracy properties, between wide limits in each. This characteristic, incidentally, is found or purposely included only in special applications of practical servomechanisms.

I do not know what the analogues are in physiology or neurology, but

it would appear that the emphasis upon stability, *per se*, is entirely an unwarranted simplification. *Degree* of stability and *degree* of accuracy-of-response would appear to be the two contrary attributes which must be considered together in extrapolating servomechanism and feedback system dynamics over into other fields of science.

Those of us engaged in the relatively simpler field of the so-called "exact sciences" of physics, mathematics, and engineering will be interestedly and expectantly following the difficult work in the largely uncharted, unmeasured fields where researchers like Dr. McCulloch are engaged. By such free and open interchange of concepts as has transpired at this conference, man's understanding, and thereby his well-being, may finally be increased.

DR. L. A. MACCOLL (*Bell Telephone Laboratories, New York, N. Y.*):

Out of the discussion of Professor McCulloch's paper, a question has arisen as to means for stabilizing feedback systems. The subject may be treated, briefly, as follows, in a manner which relates it to some of the other topics which have been dealt with at the conference.

If we have an unstable feedback system, or a system which is stable in the strict sense but not by a sufficiently large margin, there are essentially two ways in which we can go about improving the stability of the system.

In the case of a physical system with one feedback path, we can increase the stability by decreasing the amplification of the amplifier in the feedback loop. It would seem that this procedure has a parallel in the case of such biological systems as have been discussed by Professor Wiener and his co-workers, as well as by Dr. Livingston. Presumably, the administration of depressive drugs results in raising the thresholds of certain parts of the nervous system. Thus, removal of instability in a biological system by means of such drugs can be regarded, in a sense, as stabilization by decreasing the "amplification" of the part of the nervous system that is involved.

However, this procedure for securing stability is subject to severe disadvantages and limitations. Decreasing the amplification of the amplifier tends to diminish the speed and accuracy of the system, and decreasing the amplification sufficiently to make the system satisfactorily stable may result in making the system so sluggish and inaccurate as to be worthless. (The biological parallel, in the terms suggested above, is obvious.) Hence, we usually endeavor to secure stability in a basically different way.\*

The behavior of a linear system with one feedback loop is characterized by the amplification and phase shift which a sinusoidal signal under-

\* Since the procedures employed are highly technical, it is not possible to give more than a very brief and imperfect description of them here. They are discussed at length in the following publications: BODE, H. W. *Network Analysis and Feedback Amplifier Design*. Van Nostrand. New York. 1945. MACCOLL, L. A. *Fundamental Theory of Servomechanisms*. Van Nostrand. New York. 1945.

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goes when it is transmitted completely around the feedback loop. (Both characteristics, *i.e.*, the amplification and the phase shift, are to be regarded as functions of the frequency of the sinusoidal signal.) For the system to be satisfactorily stable, these characteristics have to fulfil certain technical conditions. Among these is the condition that the phase shift shall not be near one hundred and eighty degrees at any frequency at which the amplification is near unity. Broadly speaking, the practical methods for securing stability amount to modifying the system, if necessary, so that these "loop transmission characteristics" will satisfy the conditions necessary for stability.

Sometimes the necessary modifications of the system amount to nothing more than changes in the values of certain passive elements, such as inertias, stiffnesses, and resistances. In other cases, the necessary modifications can be best effected by introducing additional passive elements. In still other cases, it may be necessary to introduce subsidiary feedback loops, as has been pointed out by Professor Wiener in his remarks concerning the problem of steering a ship. The procedure is extremely flexible, and the skilful exploitation of the manifold possibilities usually leads to good results.

This general procedure for securing stability also has an evident parallel in the biological field.

Let us consider, for example, a person learning to skate. We observe that, at first, he tends to hold large parts of his body more or less rigid, and that he makes use of only a few rather simple and slow motions. He is slow to detect an incipient catastrophe, and when he does detect it he is apt to overcorrect in his attempts to avoid it. As a result, his stability is deplorable. In the course of time, however, he learns how to detect incipient catastrophes more promptly, and how to correct for them by means of a variety of small and rapid motions. As a consequence, his stability is increased. From the dynamical point of view, the fact that he is making use of a greater variety of possible motions means that he has learned how to introduce additional degrees of freedom into the system. This increase in the complexity of the dynamical system is accompanied, of course, by a corresponding enlargement of the part of the nervous system that is concerned in the process. Presumably, there is a large increase in the number of feedback loops in the total neuro-mechanical system. Although the situation is very complicated and difficult to analyze in detail, it is quite apparent that we have here an analogue of the case of an engineer stabilizing an unstable servomechanism by making alterations of the kind outlined above in the structure of the system.













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THYROID FUNCTION AS DISCLOSED BY  
NEWER METHODS OF STUDY\*

*Consulting Editor: J. H. MEANS*

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# INTRODUCTION TO THE CONFERENCE

By J. H. MEANS

*Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts*

WHEN, nearly a year ago, I received an invitation from Dr. Ross F. Nigrelli to prepare a program for a conference on *Thyroid Action and Anti-Thyroid Drugs*, I accepted with enthusiasm. However, I suggested that the title of the conference be changed to *Thyroid Function as Disclosed by Newer Methods of Study*. It seemed to me that it would be more profitable to discuss several new approaches to thyroid function than limit the discussion to anti-thyroid drugs alone.

Previous to the Conference, I received a telegram from Jane Stafford of Science Service asking for the gist of my introductory remarks, and hoping that I would include a statement on present or future practical aspects of current thyroid research. I wired back that the purpose of the Conference is to explore fundamental physiology of the thyroid gland, and not to concern itself with practical applications. Even to one like myself, who by training can approach thyroid problems only on the clinical level, as the years go by their fundamental aspects become increasingly intriguing. The make up of the program gives assurance that the objective will be reached. Among the contributors we find anatomists, biochemists, physiologists, pharmacologists, and clinical endocrinologists. All are distinguished scientists who have explored, by various routes, the deep secrets of the thyroid. Unquestionably, the work of each is familiar to all the others, but it is my expectation that, by coming together in this way, the fecundity of aggregation, as Walter Cannon loved to call it, may become operative and that these scientists, by their deliberations, may advance our subject further than if they had not come together here. There are present, too, not listed in the program, others who are equally qualified to contribute to the Conference. I hope that they will take part in the discussion.

It is quite fitting to have a meeting of this kind just now because, in recent years, new methods of investigation have made possible the accumulation of much new knowledge. Among such methods, or approaches, may be mentioned tracer studies by means of radioactive iodine; modification of thyroid function, at will, by means of anti-thyroid or goitrogenic drugs; the techniques of enzyme chemistry; histo- and cyto-chemical methods; tissue culture techniques; microdissection; hormone assays (bio- and chemical); and doubtless others.

What questions are we seeking to answer by such methods? It seems to me they have to do with such matters as the mechanism of thyroid hormone synthesis; the storage, delivery, and transport of thyroid hormone; the agencies through which these functions are controlled; how the thyroid hormone reaches and impinges upon its end-organs, the cells

of the body; how it affects the end-organs; and what becomes of the hormone after it has exerted its action.

Then, coming to the pituitary hook-up, we want to explore these same functions as they relate to the thyrotropic hormone. For instance—What also is the rate and manner of secretion of this hormone? How is it controlled? How does this hormone act on its end-organ—the thyroid cell? and, How is the whole pituitary thyroid axis controlled from without; that is to say, through the nervous system, or through the influence of other of the endocrine glands?

Some of these questions will be answered by our contributors, others will be debated—perhaps clarified—though not answered.

There is one approach that I have not yet mentioned. That is the phylogenetic. It is one of which I know but little, though it rather fascinates me. I am curious about how we acquired these endocrine glands which we are discussing, and I wonder if an understanding of their evolution may not contribute to our knowledge of their present functioning. Was the thyroid once a gland of external secretion? If so, how did it get changed to one of internal secretion? Why is it that, whereas most of the endocrine glands of the present-day vertebrate appear to be under the control of the pituitary, the parathyroids and (as I learned from Dr. Roy Greep the other day) the glomerulosa portion of the adrenal cortex seem not to be under pituitary control? What signifies it, moreover, that while the thyroid is chiefly under pituitary control, it also seems, to some degree, directly to control itself? Do those endocrine activities which are not controlled by the pituitary represent older functions than those that are under pituitary control? I have been curious for years about the endocrinology of invertebrates, but, alas, never had the training to explore it. There seems to be rather scant information on the subject. The first I ever got was from the 1945 Dunham Lecturer at Harvard, Dr. Vincent B. Wigglesworth, Director of the Unit of Insect Physiology of the London School of Tropical Medicine. In some very beautiful experiments on a certain tick, he showed very clearly that hormones of its own play a role in both the growth and maturation of this insect.

I think a great deal can be done in the field of comparative endocrinology. If I had the money, I would found a department of comparative endocrinology, but I have not. However, because of these interests I was delighted to be able to induce Dr. E. D. Goldsmith of New York University to open our program with a talk on the phylogeny of the thyroid gland.

# PHYLOGENY OF THE THYROID: DESCRIPTIVE AND EXPERIMENTAL\*

BY F. D. GOLDSMITH

*Department of Histology, College of Dentistry, New York University,  
New York, N. Y.*

IN NATURE'S INFINITE BOOK OF SECRECY  
A LITTLE I CAN READ.

*Shakespeare*

IN the preface to his excellent volume, *Our Face from Fish to Man*, Dr. William King Gregory<sup>1</sup> states that "this book does not pretend to tell how to improve one's face but only how and why one has one." In the same vein, the following is a presentation of a few facts, some descriptive and others pertaining to the experimental, as to the origin and relationship of the thyroid gland in the animal series. The study of function, mechanism, and treatment of glands running wild is left to the authors who follow.

## *Descriptive*

In the human, clasping the upper end of the trachea, lies the thyroid gland, first accurately described and named by Thomas Wharton<sup>2</sup> in 1659. It arises early in fetal life, toward the end of the fourth week, by a downgrowth of epithelium from the ventral wall of the pharynx between the first and second pharyngeal arches (FIGURE 1a). With the forward growth of the pharynx, the gland primordium appears to grow caudally, dividing into a right and left lobe, remaining attached to the pharynx by a stalk or neck, the thyroglossal duct. This structure temporarily connects the young thyroid with the posterior border of the developing tongue. During the fifth week, the thyroglossal duct atrophies, but its site of origin remains as the *foramen caecum* in post-uterine life. Segments of the thyroglossal duct which do not become obliterated may give rise to thyroglossal cysts (FIGURE 1b).

Prior to the development of the various branches of experimental biology, much thought and effort were expended in the study of the origin of the vertebrates and interrelationships of the invertebrates. Examination of the phylogenetic tree (FIGURE 2) reveals the animal relationships as derived from the descriptive evidence presented by the embryologist and morphologist.

As we descend the tree, we find that development of the thyroid gland is similar, with minor exceptions, in the various mammals (man,<sup>3, 4</sup> pig,<sup>5, 6</sup> calf,<sup>7</sup> horse,<sup>8</sup> dog,<sup>9</sup> rat,<sup>10, 11, 12</sup> guinea pig,<sup>13</sup> mole,<sup>14, 15</sup> bat,<sup>16</sup> *Tar-*

\* The original research here cited of the author and his associates was aided by grants from the Commonwealth Fund, Josiah Macy, Jr., Foundation, and the Elizabeth Thompson Science Fund. The colored plates were generously supplied by Dr. John R. Mote, Medical Director, The Armour Laboratories, Armour and Company.

*sius*<sup>17</sup>); in birds,<sup>18-22</sup> in reptiles,<sup>23, 24</sup> and Amphibia.<sup>25-29</sup> The basic similarity is exemplified in a urodele, *Necturus*, where the thyroid arises as a single median diverticulum from the pharynx, and grows caudally, splitting into a right and left lobe and leaving an anterior median mass.<sup>29-32</sup> In the adult *Necturus*, Charipper<sup>33</sup> has described the thyroid as consisting of three masses, the more anterior median one often connected to the lateral masses by a tract of follicles reminiscent of the persistent thyroglossal duct.

In *Squalus acanthias*, an elasmobranch, the thyroid arises as a solid epithelial bud in the region ventral and caudal to the ventral borders of the first and second gill pouches in embryos approximately 4 mm. in length, with follicles developing as they do in the human.<sup>34, 35</sup> The gland itself is said to be encapsulated. As for the thyroglossal duct, it was reported in *Squalus acanthias* by W. Müller<sup>36</sup> and in a primitive selachian, *Chlamydoselachus*, by Goodey,<sup>37</sup> but was not observed in *Squalus* by Norris.<sup>35</sup>

In a ganoid, *Amia*,<sup>38</sup> the thyroid primordium arises as a solid median outgrowth from the pharynx, becoming detached early in development (FIGURE 3), leaves no thyroglossal duct, and migrates to a position between the bases of the third visceral pouches ventral to the aorta. In the adult fish, the gland consists of a group of follicles scattered in the ventral pharyngeal region around the aorta.<sup>38</sup> Hill associated the thyroid primordium with the region of the first and second visceral pouches, corresponding thus to the location of the thyroid primordium in the teleosts studied by Maurer.<sup>39</sup>

The thyroid of the teleost was first reported in 1844 by Simon,<sup>40</sup> who erroneously believed it to be absent in the salmon and trout. The teleost thyroid has been described<sup>39, 41-45</sup> as constituted of numerous units, the follicles scattered over a wide area. In its development, the trout thyroid arises as a vesicular evagination from the floor of the pharynx in the region between the first and second gill pouches.<sup>39</sup> From his comprehensive survey of the thyroid gland in the teleost, Gudernatsch<sup>41</sup> arrived at the same conclusion. More recently, Hoar,<sup>42</sup> in his studies of the Atlantic salmon, found that the thyroid anlage develops as a solid knob-like structure from the mid-ventral floor of the pharynx at the posterior margin of the hyomandibular junction. These data are in agreement with those reported for the dipnoan, *Ceratodus*.<sup>46</sup> With regard to the capsule present in the elasmobranchs and in all higher forms, it should be noted that there is a very great range in the degree of compactness of the thyroid tissue in the teleosts.<sup>41, 42</sup> More specifically, in the salmon, *Salmo salar*, in very large individuals, the acini may be crowded together to form a compact and nodular gland.<sup>45</sup>

(See opposite page)

FIGURE 1a. The development of the thyroid gland. (From *The Thyroid Gland*, The Armour Laboratories, 1943. Drawings by Dr. Frank Netter.)

*Cyclostomes and Protochordates.* Upon examination of the adult cyclostomes, one finds a thyroid gland consisting of a small number of isolated follicles.<sup>47-53</sup> This is the most primitive thyroid, and a study of the life history of the cyclostomes reveals several interesting facts. Prior to its becoming an adult, the cyclostome passes through a larval stage. To the larva of the cyclostome, *Petromyzon*, the name *Ammocoetes* was given in the mistaken belief that it was an adult member of a distinct genus. The general appearance of this larva is somewhat like that of another organism lower in the phylogenetic series, *Amphioxus*. Both burrow on the bottoms of streams and feed on microscopic organisms. In *Amphioxus* (FIGURE 4) and in a related protochordate, the ascidian or tunicate (FIGURE 5), the mid-ventral line of the pharynx is marked by conspicuous grooves, the hypobranchial groove or endostyle. It is of pharyngeal origin (FIGURE 6), is composed of cuneiform cells, and is believed to secrete mucus. The food is driven into the pharynx by cilia, is entangled in a mass of mucus secreted by the endostyle,\* and is then passed by cilia on the gill bars to the esophagus.

In the *Ammocoetes* larva, below the first to fifth gill arches, there is what appears to be a similar groove which arises as a trough in the floor of the pharynx. This structure, too, has been called "endostyle." Arising from the floor of the pharynx, it pinches off, remaining connected during larval life by a duct opening between the third and fourth gill pouches. Alcock<sup>54</sup> studied the digestive effects of a number of *Ammocoetes* tissues on fibrin, but could find no proteolytic ferment in the endostyle. Hyman<sup>55</sup> is of the opinion that this organ does not resemble the endostyle of protochordates and appears not to function in feeding. As the result of a careful survey, Leach<sup>56</sup> concludes that "no comparable anlage of definitive thyroid follicles exists in the endostyle of the protochordates."

Call this structure what you will, endostyle or hypobranchial groove, we do know that it persists for 3 or 4 years and then, at metamorphosis, first described by A. Müller<sup>57</sup> in 1856, it undergoes involution with but a small portion remaining. During this period, it undergoes a series of profound changes which affect the various cellular components. W. Müller<sup>58</sup> compared the endostyle of the tunicates, *Amphioxus* and *Ammocoetes*, suggesting that the thyroid gland of the adult chordate was homologous to it. This point of view was supported by Anton Schneider.<sup>59</sup> As a result of a careful study of the lamprey, *Ammocoetes branchialis*, Mariné<sup>60, 61</sup> reported a progressive decrease in the size of the endostyle chambers, with changes in epithelial cells resulting in the formation of up to four ductless follicles in each half of the endostyle from type IV

\* Up to the time of Galen, it was believed that the thyroid gland aided in the lubrication of the pharynx and trachea.

(See opposite page)

FIGURE 1b. Sites of aberrant thyroid tissue. (From *The Thyroid Gland*, The Armour Laboratories, 1943. Drawings by Dr. Frank Netter.)



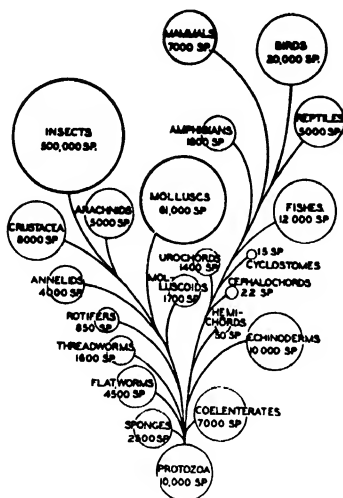


FIGURE 2. Phylogenetic tree. (From NEAL & RAND, *Comparative Anatomy*, Blakiston Co.)

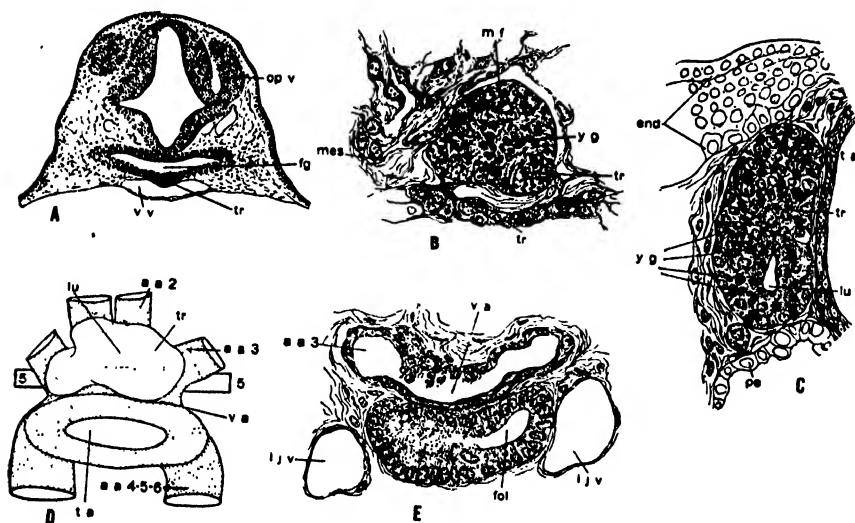


FIGURE 3. (After B. H. HILL.<sup>34</sup>) A. Frontal section through thyroid primordium of *Amia* 1 day old. B. Transverse section through thyroid of *Amia* 1 day old. C. Sagittal section through thyroid of *Amia* 2 days old. D. Graphic reconstruction of thyroid region of *Amia* of 8 mm., ventral aspect. Dotted lines show the outline of the lumen of the thyroid. E. Transverse section through thyroid of fig. D at line 5-5.

Abbreviations: aa 1-6, aortic arches 1-6; end, endoderm; fg, foregut; fol, thyroid follicle; i j v, inferior jugular vein; lu, lumen; mes, mesenchyme; mf, mitotic figure; op v, optic vesicle; pe, pericardium; ta, truncus arteriosus; v a, ventral aorta; v v, ventral vessel; y g, yolk granule; tr, thyroid primordium.

epithelium. New follicles were said to arise by budding from the primary ones (see FIGURES 7 and 8).

In more recent papers, Leach<sup>56, 62</sup> has described the structure of the

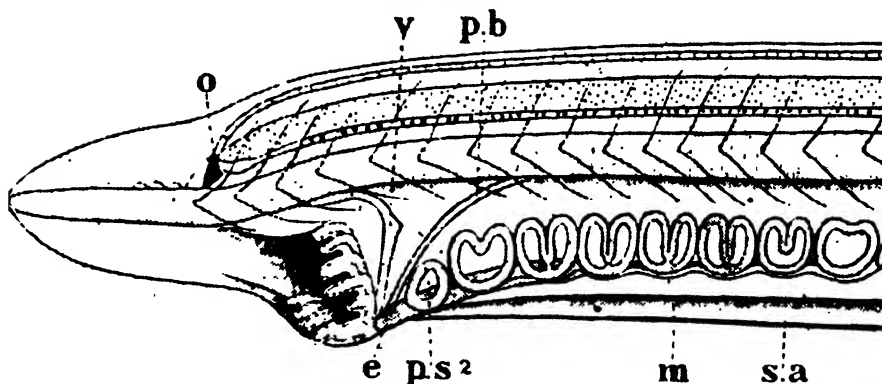


FIGURE 4. *Amphioxus* larva toward the close of metamorphosis. (From WILLEY, 1894.) *e.*, endostyle; *p.b.*, peripharyngeal band; *p.s.2*, secondary primary slit.

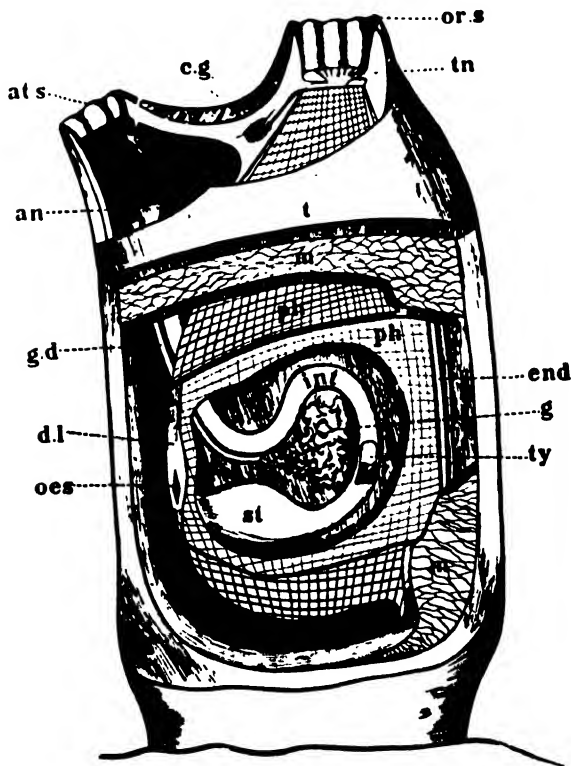


FIGURE 5. Diagram of a dissection of *Ascidia*; from the right side. (From WILLEY, 1894.) The peribranchial cavity is indicated by the black shading. *an.*, anus; *at.s.*, atrial siphon; *c.g.*, cerebral ganglion, beneath which is the subneural gland and its duct; *d.l.*, dorsal lamina; *end.*, endostyle; *g.*, gonad; *g.d.*, genital duct; *int.*, intestine; *m.*, muscular mantle; *oes.*, aperture leading from branchial sac into esophagus; *or.s.*, buccal siphon; *ph.*, branchial sac; *st.*, stomach; *t.*, test or cellulose mantle; *tn.*, buccal or coronary tentacles; *ty.*, typhlosole, internal fold of intestinal wall, to increase the digestive surface.

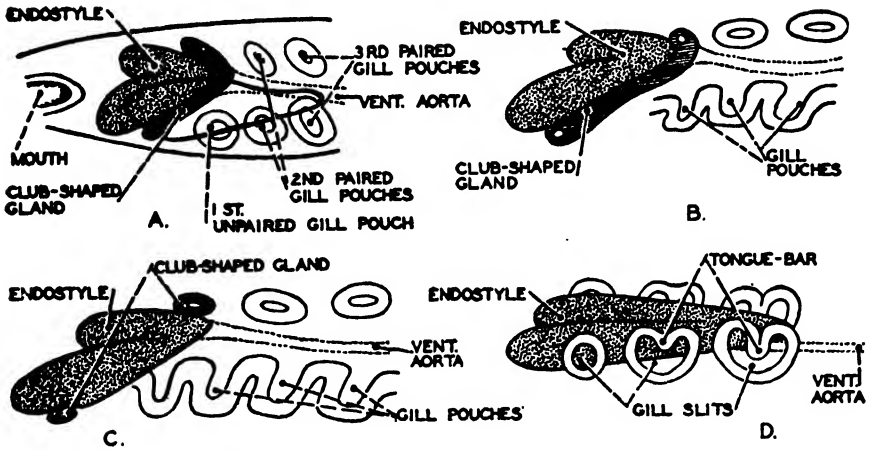


FIGURE 6. Four stages in the development of the endostyle of *Amphioxus*. The evidence indicates that the endostyle is developed from a pair of modified gill pouches. (From NEAL & RAND, *Comparative Anatomy*, Blakiston Co., after VAN WIJHE.)

endostyle (FIGURES 9 and 10) and its transformation into the thyroid gland in the brook lamprey, *Ichthyomyzon*. He recognized six types of cells in the endostyle epithelium, as compared with the five types reported by Marine. Cells of type IV appear to be most active, with type II contributing to the formation of definitive follicles (see FIGURE 11). No comparable anlagen of definitive follicles are present in the endostyle of protochordates.

Beginning, thus, with a diffuse thyroid of few follicles, the gland has become more extensive, retaining its diffuse character in the bony fishes\* and becoming encapsulated in the higher forms.

In all forms below the Chordates, direct observation has not disclosed a functional thyroid. However, Murray<sup>63</sup> in 1891 had cured myxedema by the administration of thyroid gland whose active principle was unaffected by drying and heating. It was recalled that Fyfe<sup>64</sup> had long before (1819) discovered the presence of iodine in sponges, and Coindet,<sup>65</sup> a year later, had demonstrated the therapeutic value of iodine in the treatment of goiter and had established it as the active agent in the remedies, such as seaweed and burnt sponge, which had for centuries been found beneficial in the treatment of goiter. In 1848, Vogel<sup>66</sup> showed that sponges contained iodine in organic combination. Further research<sup>67</sup> dis-

\* While this paper was in press, Matthews<sup>291</sup> described a compact encapsulated thyroid gland in 3 genera (*Pseudocarcinus guacamaia*, *Sparisoma* sp., and *Scarus* sp.) of Bermuda parrot fish. The thyroid in these fish is large, extending from a point under the base of the tongue to about a cm. anterior to the anterior end of the ventral aorta. The time of development of the capsule and/or other factors responsible for this compact gland remain to be ascertained. Previously, a compact thyroid gland for a teleost, the swordfish, *Xiphias*, had been reported by Addison and Richter.<sup>44</sup> The thyroid of *Xiphias* consists for the most part of 4 masses separated from each other by thin connective tissue septa. In addition, small groups and rows of follicles are scattered in a typical teleostean manner in the loose connective tissue between the chief portion of the gland and the wall of the aorta and the branchial vessel. It would be of interest to learn whether supplementary scattered follicles are present in the parrot fish. Since radioactive iodine was concentrated only by the thyroid gland and by the tissues of the gill region in the parrot fish,<sup>291</sup> it is very likely that thyroid follicles are to be found in this region. The final answer awaits histological study.

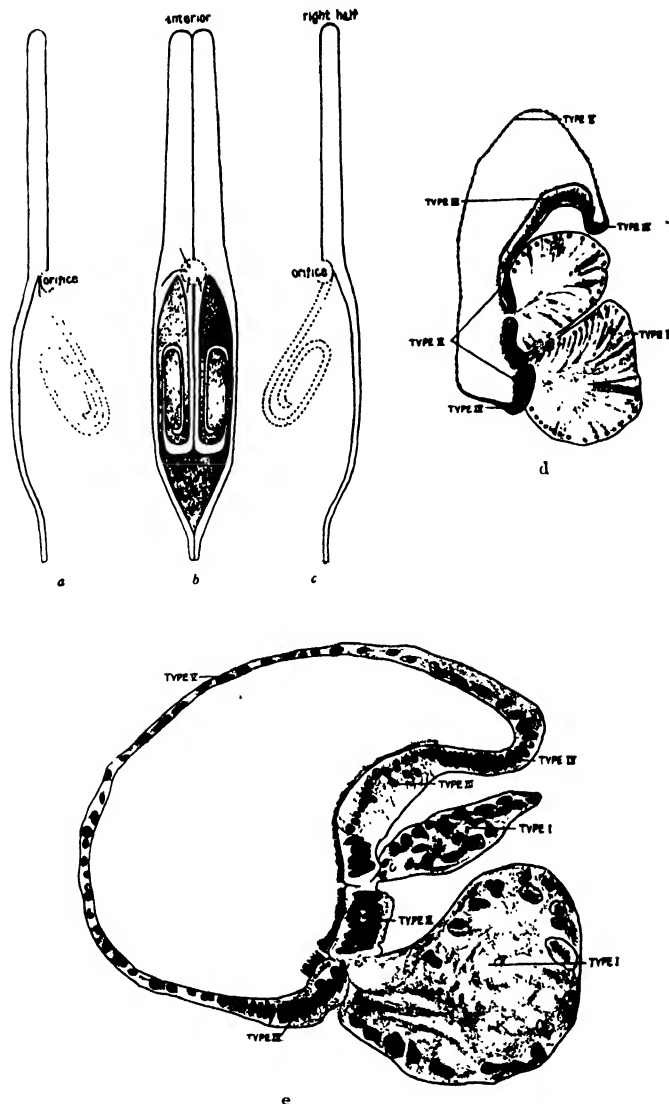


FIGURE 7. Diagrams of the endostyle of *Ammocoetes branchialis* (after MARINE<sup>41</sup>). *a*, Schematic diagram of the left half of the endostyle. The single anterior chamber branches at the anterior edge of the orifice into an inner coiled chamber and an outer coiled chamber. *b*, Composite diagram of right and left halves; the two coiled chambers rotated outward from their normal upright positions. *c*, Schematic diagram of the right half of the endostyle. *d*, Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the arrangement and distribution of five types of epithelium in the normal fully developed endostyle. *e*, Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the earliest changes in the epithelia at metamorphosis.

closed that tropical and subtropical horny sponges contained as high as 14 per cent iodine in organic combination. At this time, Baumann<sup>68</sup> re-

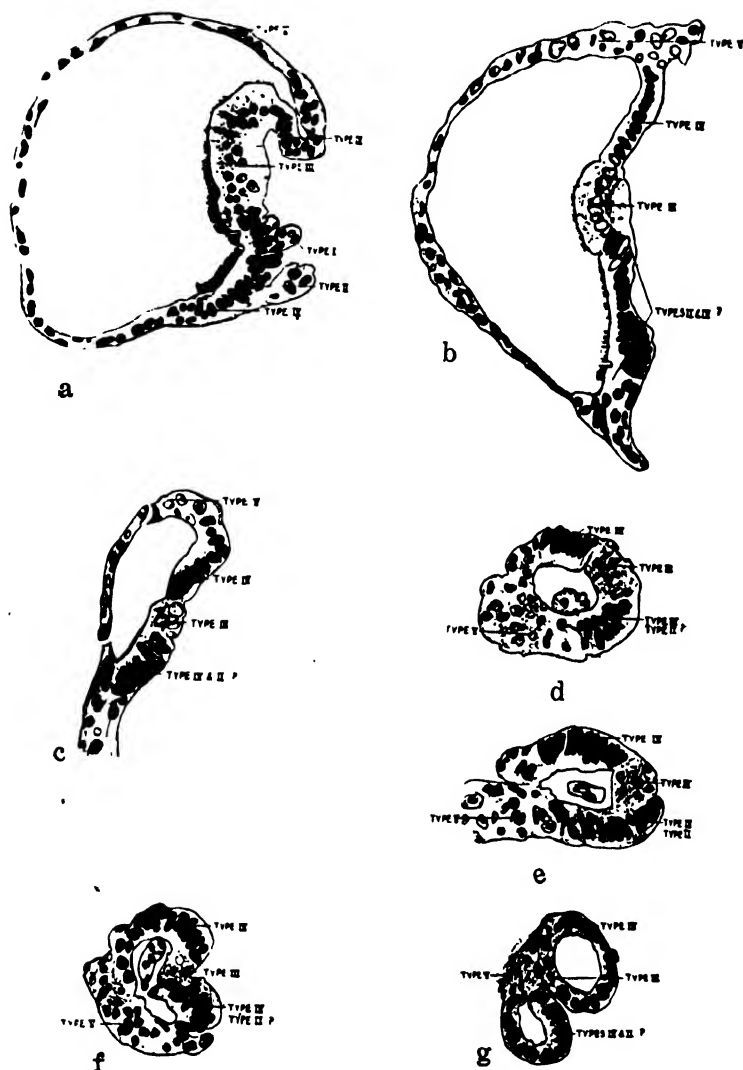


FIGURE 8. Diagrams of the development of thyroid follicles from endostylar cells. (After MARINE.<sup>61</sup>) *a, b, c*, Camera lucida outline of transverse section of left external chamber posterior to the coil showing several stages of metamorphosis. *d, e*, As above, but showing last stages of metamorphosis. *f*, As above, but illustrating the earliest ductless thyroid formation. *g*, As above, but illustrating two distinct follicle formations from single chamber.

ported that he had boiled thyroid glands with 10 per cent sulfuric acid and had separated a physiologically active fraction comprising about 0.2 to 0.5 per cent of the gland substance and containing about 10 per cent iodine. The material was named thyreiodin and then renamed iodothylin. Simultaneously, Drechsel<sup>69</sup> isolated an iodine-containing

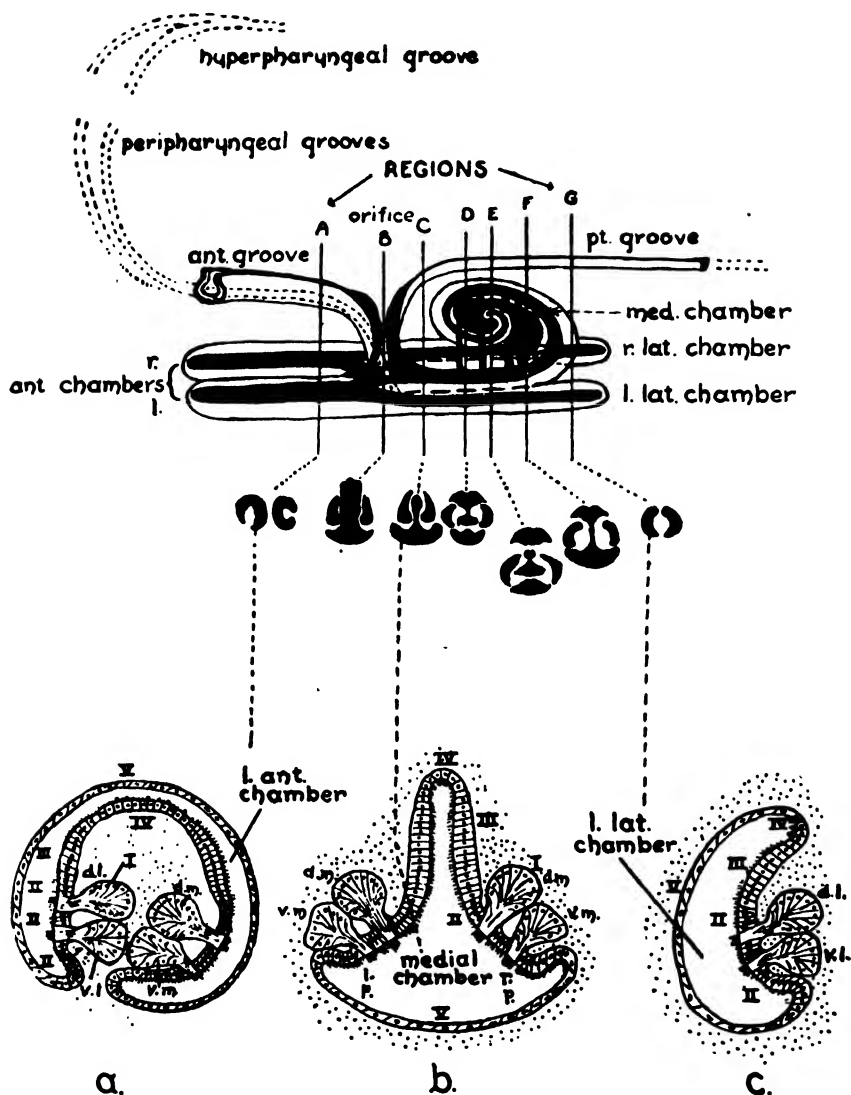


FIGURE 9. A diagram to show the structural plan of the endostyle. Endostylar chambers are shown in solid black in the upper portion of the figure, and their cross-sectional distribution below the regional lines A to G. The lower portion of the figure (a, b, c) illustrates the three fundamental types of chambers in relation to the distribution of the types of cells (I to V). *d.l.*, dorsolateral cylinder; *v.l.*, ventrolateral; *d.m.*, dorsomedial; *v.m.*, ventromedial (bilateral aspects are shown only in b); *l.p.*, left pair of cylinders; *r.p.*, right pair. (After LEACH.<sup>56</sup>)

amino acid—iodogorgonic acid or iodoaminobutyric acid. Following Henze,<sup>70</sup> who had indicated that the substance was not iodoaminobutyric, Wheeler and Jamieson<sup>71</sup> demonstrated that it was 3,5-diiodotyrosine. Similarly, this was also shown to be the iodine complex in the sponge.<sup>72</sup>

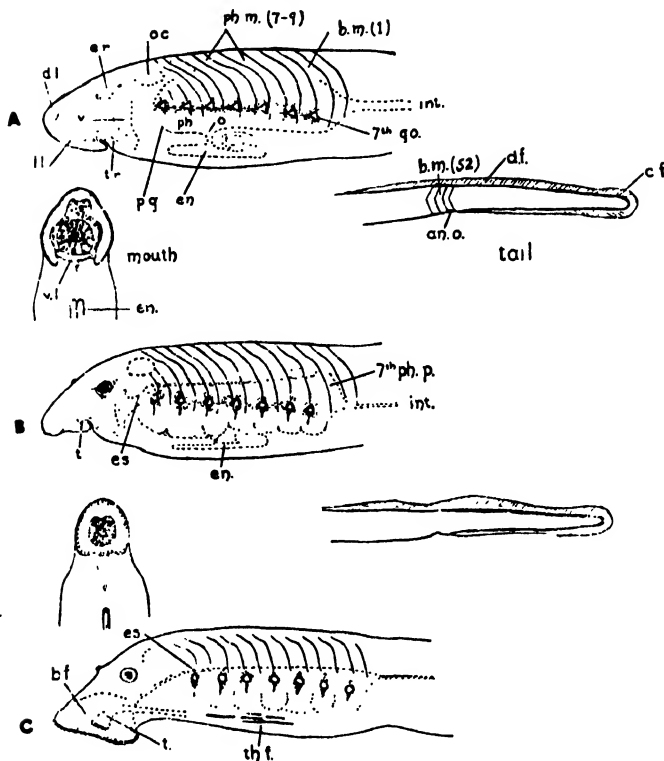


FIGURE 10. The transformation of the brook lamprey, *Ichthyomyzon fossor*. A, Morphological features of a fully grown ammocoete. B, Changes during early metamorphosis. C, Morphological condition of sexually mature male. *an.o.*, anal opening; *b.f.*, buccal funnel; *b.m.*, body myotomes; *c.f.*, caudal fin; *d.f.*, dorsal fin; *d.l.*, dorsal lip; *en.*, endostyle; *es.*, esophagus; *er.*, eye rudiment; *int.*, intestine; *l.l.*, lateral lip; *o.*, orifice of endostyle; *o.c.*, otic capsule; *ph.m.*, pharyngeal myotomes; *p.g.*, peripharyngeal grooves; *t.*, tongue; *th.f.*, thyroid follicles; *t.r.*, tongue rudiment; *v.l.*, ventral lip; *7th g.o.*, seventh gill opening; *7th ph.p.*, seventh pharyngeal pouch. (After LEACH.<sup>62</sup>)

Since that time, iodine analyses<sup>73, 74</sup> have disclosed iodine in all marine animals ranging from traces to as high as 78 gms. per kilogram in the skeleton of the sea fan.

### Experimental

In order to ascertain whether animals which normally do not possess a thyroid gland respond to its secretions, it was necessary to resort to experimental techniques. Since the investigations conducted on the invertebrates and protochordates were influenced by those performed upon the Amphibia and mammals, it might be profitable to step out of phylogenetic line and to recall several critical experiments.

Gudernatsch,<sup>75</sup> some 35 years ago, maintained frog tadpoles on a variety of diets and discovered that fresh thyroid glands induced an amazingly accelerated metamorphosis. The hypophysectomy of the frog by

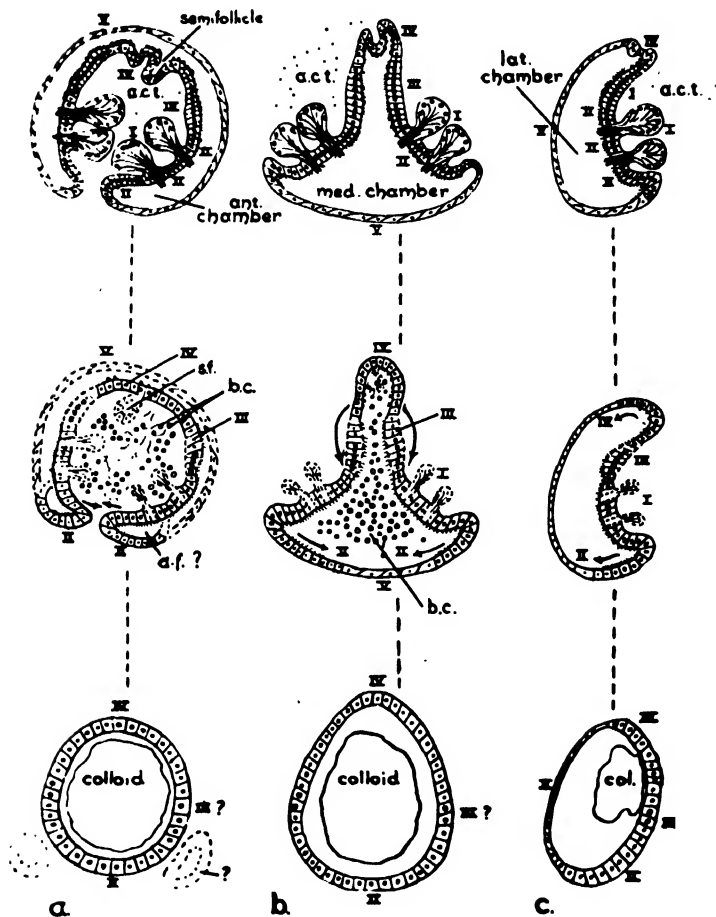


FIGURE 11. The essential features of the development of definitive thyroid follicles. Upper row, diagrams of endostylar chambers of the arrested growth period; middle row, at the end of the histolysis phase of early transformation; lower row, definitive follicles of the sexually ripe adult. *a.c.t.*, areolar connective tissue; *a.f.*, an aberrant type of follicle sometimes seen in early transformation; *b.c.*, blood cells assumed to migrate to the medial follicles; *a.f.*?, indicates the fate of the semi-follicles; arrows indicate the realignment of the cell types. (After LEACH.<sup>50</sup>)

P. E. Smith<sup>76</sup> and hypophysectomy and thyroidectomy by B. M. Allen<sup>77-79</sup> gave us the now well-known picture of a tadpole which would not go through the various maturation changes leading to the adult frog.

The results of the original feeding experiments of Gudernatsch were so startling that one is apt to forget the picture in the mammal. The failure of the cretin to attain the status of what we regard as the normal adult is evidence in favor of the view that the thyroid plays an important role in the maturation of the mammal. This point Gudernatsch has repeatedly emphasized. With these facts for a background, one may proceed to the experimental phase of the review.



*Invertebrates.* Examination of the data in TABLE 1 reveals that exposure of Infusoria to thyroxin, thyroid extracts, or thyroid powder yielded controversial results. Cori<sup>83</sup> has suggested that thyroid extract

TABLE 1  
SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION TO INFUSORIA

<i>Investigator</i>	<i>Substance</i>	<i>Results</i>
Nowikoff <sup>80</sup>	Thyroid powder	Accelerated cell division
Shunway <sup>81</sup>	Fresh thyroid powder	Accelerated cell division
	Iodine, iodothyrim	No effect
Buddington & Harvey <sup>82</sup>	Fish, frog, turtle, chick, cat thyroid	Accelerated cell division
Cori <sup>83</sup>	Thyroid extract	Accelerated cell division
	Thyroxin 1:100,000	Questionable
Woodruff <sup>84</sup>	Thyroid powd., thyroxin	No effect
Ball <sup>85</sup>	Thyroid extract	Accelerated cell division
Torrey <sup>86</sup>	Thyroxin 1:100,000 - 1:1,000,000	Depressed cell division Accelerated catabolism

may produce its effect in the invertebrates through some principle other than thyroxin. In his review paper, Schneider<sup>87</sup> concluded that thyroid extract and thyroxin exert an effect on the metabolic processes in the unicellular organism and during the early cleavages of other invertebrates.

Thyroid feeding in *Planaria velata* resulted in an apparent speeding-up of metabolic processes and a great reduction in size. This decrease was not due to inanition, since the animals were observed taking the food readily. Moreover, they became smaller than those which were starved.<sup>88</sup> In another species of flatworm, *Planaria maculata*, Goldsmith<sup>89</sup> observed that thyroid feeding did not bring about a reduction in size nor did it alter the rate of head regeneration following a number of successive decapitations. It was, however, observed that the worms on the thyroid diet produced significantly fewer egg capsules than did the animals on other solid diets (TABLE 2).

Little has been done with the annelids. Young polychaete worms were treated with thyroid gland preparations and appeared to show a precocious sexual maturity including the more rapid development of ripened eggs. Growth and regeneration were not affected. Inorganic iodine yielded negative results.<sup>90</sup>

The literature pertaining to the effects of thyroid principle upon insects is replete with contradictions and inconsistencies. The partial summary (TABLE 3) presents the state of our knowledge in this connection. Much of the experimentation is open to criticism in that dosages were not adequately controlled (possible improvement of the ration by the

thyroid supplement or possible toxicity of higher concentrations) and the insects were not of known ancestry. In one of the better conceived statistical investigations, Alpatov<sup>102</sup> concluded that thyroid feeding ac-

TABLE 2

NUMBER OF EGG CAPSULES PRODUCED BY *PLANARIA MACULATA* ON INDICATED DIETS

Number of animals	Food	Number of capsules to 4/22*	Number of capsules to 5/12
9	Liver gland	68	87
18	Anterior pituitary gland	36	40
17	Anterior pituitary-thyroid gland	55	62
18	Thyroid gland	18	20
4	Liver aqueous extract L	1	1
5	Liver aqueous extract S	0	0
9	Anterior pituitary aqueous extract	0	0
9	Anterior pituitary-thyroid aqueous extract	0	0
9	Thyroid aqueous extract	0	0
9	Starve	0	0

\* The animals were decapitated on 4/23. They continued to produce capsules to 5.12. (From GOLD-SMITH, E. D. Biol. Bull. 73.)

celerated larval development, with smaller adult *Drosophilae* emerging. In a thorough study of *Tribolium*, Schneider<sup>107</sup> selected optimum dosages of thyroid substance and was able to accelerate metamorphosis of not only the treated larvae but also the untreated larvae of the F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> descendants.

The work with the crustaceans, molluscs and echinoderms, too, does not enable one to reach a satisfactory conclusion. The bulk of the evidence appears to favor the view that thyroxin does not benefit or accelerate development (TABLE 4).

*Ascidians.* As was pointed out in the "Descriptive" portion of the paper, the ascidian, a protochordate, possesses an endostyle, passes through a larval stage, and undergoes metamorphosis. In the adult, no thyroid gland is recognizable. Here, then, appeared excellent material to test the effects of thyroid substance (TABLE 5). To this end, Weiss,<sup>121</sup> using sea-water solutions of thyroid extracts, hastened the resorption of the tail in *Ciona intestinalis*. That thyroid substance was not specific for this acceleration is to be concluded from subsequent work. 100 per cent metamorphosis was induced in groups of *Ascidia nigra* larvae within 3 hours after hatching by sea-water extracts of pharyngeal, atrial or mantle tissues of adult *A. nigra*, as compared with 30 hours for 100 per cent metamorphosis in untreated controls.<sup>122</sup> The acceleration was even greater in another form, *Polyandrocarpa*. Sea-water extracts of mammalian whole thyroid glands also accelerated metamorphosis, as did cysteine, histidine, leucine, iron and copper salts, and iodine to a lesser degree.<sup>123</sup> Diiodotyrosine, thyroxin, *dl*-phenylalamine, and cysteine yielded negative results. Sea-water extracts from ascidian endostyles acted as very efficient accelerators, but not more so than other ascidian tissues. Brad-

TABLE 3

## SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION TO INSECTS

<i>Investigator</i>	<i>Animal</i>	<i>Substance</i>	<i>Results</i>
Kunkel <sup>91</sup>	<i>Lucilia, Calliphora</i>	Mammalian thyroid excl.	Short. larval & pupation
Romeis <sup>92</sup>	<i>Calliphora</i>	Mammalian thyroid excl.	Retardation
Brannon <sup>93</sup>	<i>Lucilia</i>	Mammalian thyroid excl.	Short. pupation
Abderhalden <sup>94</sup>	<i>Deilephila</i>	Hydrolyzed thyroid	No effect, great variation
Kopeč <sup>95</sup>	<i>Lymantria, Pieris</i>	Willow leaves & thyroid	No effect, smaller chrysalids
Magaudda <sup>96</sup>	<i>Pieris</i>	Thyroid extracts	Short treatment - acceleration Long treatment - retardation
Romeis <sup>97</sup>	<i>Papilio</i>	Inject thyroxin	No effect
Fleischmann <sup>98</sup>	<i>Lymantria</i>	Inj. thyrox. 1:10,000	No effect
Fleischmann <sup>98</sup>	<i>Celerio</i>	Inj. 0.3 mg. thyroxin	No effect
Kahn <sup>99</sup>	<i>Tenebrio</i>	Thyroid substance	No effect
Hahn <sup>100</sup>	<i>Tenebrio</i>	1 thyroid: 3 meal	No effect on rate or size
Hahn <sup>100</sup>	<i>Vanessa</i>	Nettle leaves & thyroid	No effect on rate or size
Reznitchenko <sup>101</sup>	<i>Drosophila</i>	Thyroid 5% ration	No effect
Reznitchenko <sup>101</sup>	<i>Drosophila</i>	Thyroid 10% ration	Retardation
Alpatov <sup>102</sup>	<i>Drosophila</i>	Thyroid in ration	Short larval, smaller
Koller <sup>103</sup>	<i>Drosophila</i>	Thyroxin	No effect
Comas <sup>104</sup>	<i>Chironomus</i>	Thyroid extract	Short larval
Zavřel <sup>105</sup>	<i>Chironomus</i> late summer	Thyroid extract	No effect on metabolism
Zavřel <sup>105</sup>	<i>Chironomus</i> early summer	Thyroid extract	Accel. growth & metab.
Terao & Wakamori <sup>106</sup>	<i>Bombyx</i>	Mulberry leaves & thyr. 1:50 1:100 food	{ Ate less. Adults smaller. Accel. 2nd generation
Schneider <sup>107</sup>	<i>Tribolium</i>	1 thyroid: 5 food 1:10 1:100	No effect Accel. to 2nd generation

way<sup>124</sup> obtained no speeding up with crystalline thyroxin in sea-water solutions of 1:10,000, 1:20,000, down to 1:1,280,000. Immersion of larvae in (1) isotonic sodium chloride alone at pH 8.2 for one hour followed by normal sea-water, (2) sea-water diluted 50% by distilled water for 15 minutes and then transfer to normal sea-water, or (3) sea-water containing neutral red 1:50,000 was followed by marked acceleration of metamorphosis. Additional evidence of the non-specificity of the stimulating agent is to be found in the observations of Zinkin<sup>125</sup> in another tunicate, *Botryllus*, in which brilliant cresyl blue, methylene blue, neutral red, strychnine, and potassium bicarbonate in certain concentrations accelerated metamorphosis. These results are summarized in TABLE 5.

Corroborating these findings from another angle, Gorbman<sup>126</sup> has demonstrated that in the ascidians, *Styela* and *Ciona*, as well as in *Amphioxus*

TABLE 4  
SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION  
TO CRUSTACEA, MOLLUSCS AND ECHINODERMS

<i>Investigator</i>	<i>Animal</i>	<i>Substance</i>	<i>Results</i>
<i>Crustacea</i>			
Vecchi <sup>108</sup>	<i>Cyclops</i>	Dried thyroid	Accelerated development
Banta <sup>109</sup>	<i>Daphnia</i>	Dried thyroid	No effect
Romeis <sup>110</sup>	<i>Astacus</i>	Thyroid excl. 5 mo.	No effect. Transf.
Chiabetta <sup>111</sup>	<i>Artemia</i>	Thyroid extracts	Accel. develop. small
<i>Mollusca</i>			
Hykès <sup>112</sup>	<i>Physa</i>	Thyroxin 1:100,000-1:500,000	Uncertain
Susaeta <sup>113</sup>	<i>Barnea</i> eggs	Thyroxin, low & high	Retard. development
Susaeta <sup>113</sup>	<i>Barnea</i> eggs	Thyroid 5 cc/liter	Slight acceleration
Susaeta <sup>113</sup>	<i>Barnea</i> eggs	Thyroid 5 cc + /liter	Retard. 2 & 4 cell suscep.
Chatzillo <sup>114</sup>	<i>Limnaea</i>	Thyroid	Accel. develop. in 60%
<i>Echinoderm</i>			
Butler <sup>115</sup>	<i>Arbacia</i>	Thyroxin 1:25,000	Retard. 1st cleav. 10 min.
		Thyroxin 1:50,000	Retard. 1st cleav. 5 min.
		Thyroxin 1:100,000	Retard. 1st cleav. 5- min.
Torrey <sup>116</sup>	<i>Echinometra</i>	Thyroxin 1:50,000-1:1,000,000	Slight to comp. inhibition
Runn- ström <sup>117</sup>	<i>Antedon</i>	Thyroxin	Retardation of cleavage
Zavrel <sup>118</sup>	<i>Paracentrotus</i>	Thyroxin	Retardation of cleavage
Ungar <sup>119</sup>	<i>Psammochinus</i>	Thyroxin	No effect 1st cleav. stages
Ungar <sup>119</sup>	<i>Psammochinus</i> <i>morula</i>	Thyroxin 1:50,000-1:800,000	Retarded development
Ungar <sup>119</sup>	<i>Psammochinus</i> <i>morula</i>	Thyroxin 1:850,000-1:2,000,000	Accel. development

TABLE 5  
SUMMARY OF EXPERIMENTATION ATTEMPTING TO  
ACCELERATE METAMORPHOSIS IN ASCIDIANS

<i>Investigator</i>	<i>Recipient</i>	<i>Treatment</i>	<i>Result</i>
Spaul <sup>120</sup>	Frog	Ascidian endostyle, pharynx, mantle	--
	Frog	Dogfish thyroid	+
Weiss <sup>121</sup>	Ascidia	Immersion & feeding thyroid	+
Grave <sup>122</sup>	Ascidia	Sea-water extract pharyngeal, atrial, mantle tissue of adult Ascidia	+
	Ascidia	Sea-water extract mammalian thyroid	+
Grave & Nicol <sup>123</sup>	Ascidia	Cysteine, leucine, histidine	+
		Cysteine, tyrosine, diiodotyrosine, <i>dl</i> -phenylalanine	—
		Fe, Al, Cu, I	+
		Sea-water extract ascidian endostyle, other tissues	+
Bradway <sup>124</sup>	Ascidia	Thyroxin 1:10,000, 1:20,000 to 1:1,280,000	—
		I 1:80,000,000	+
		Immersion 15 min. sea-water dil. 50% distilled	+
		Neutral red in sea-water 1:50,000	+
Zinkin <sup>125</sup>	Ascidia	Brilliant cresyl blue, methylene blue, neutral red, strychnine, potassium bicarbonate	+

the endostyle did not accumulate radioactive iodine. Iodine was picked up and stored in the stolonian septum (FIGURE 12) to a degree fully as great

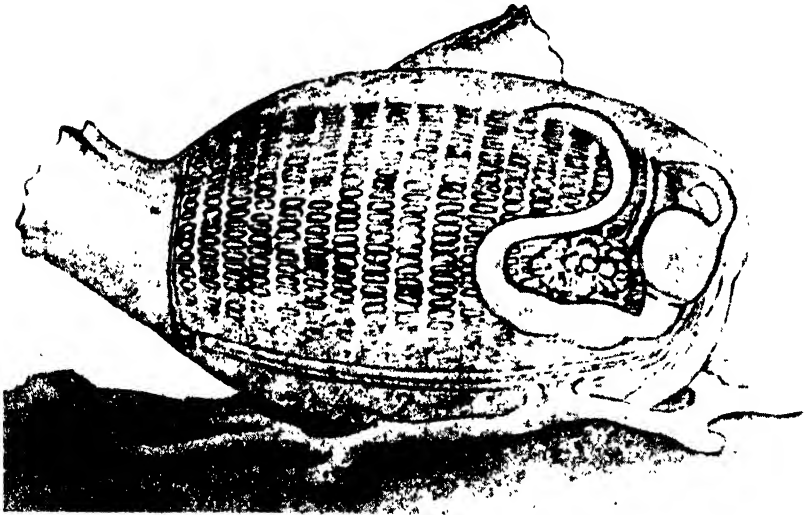


FIGURE 12. Ascidian, *Ecteinascidia*, from left side. The growing stolon is shown at base.

as the vertebrate thyroid.<sup>127</sup> Previously, Marine<sup>60</sup> had found 3.63 mg. iodine per kilogram in the tunic, with 0.03 mg. per kilogram in the rest of the body, but none in the ascidian endostyle. The significance of the high concentration of iodine in the stolonian septum and tunic escapes us at this time.

*Cyclostomes.* Considerable evidence of a morphological nature has been presented in support of the view (see "*Descriptive*") that the lamprey thyroid arises from the larval hypobranchial groove. Additional evidence is to be found in the radioactive iodine work of Gorbman and Creaser.<sup>128</sup> The lamprey, *Entosphenus*, localized radioactive iodine in the endostyle and kept it there for one to five years, i.e., for the duration of the larval period. It is of interest to note that the type III cells were found to be the principal accumulators of iodine, with smaller amounts demonstrable in type V, and negligible quantities or none at all in types I, II, and IV (FIGURE 13). The reason for the discrepancy between these observations and those of Marine and Leach is not at all clear. In FIGURE 14, one may see the uptake of radioactive iodine by the developing thyroid gland of the frog.<sup>129</sup>

Spurred on by findings in the Amphibia and developments in the treatment of myxedema, attempts were made to alter the rate of metamorphosis of the lamprey by exposure to substances of known potency. A number of *Ammocoetes* had been immersed in sea-water containing

Lugol's solution, but no modification of the endostyle could be recognized.<sup>60</sup> Analyses of ammocoete endostyles by Kendall's method,<sup>130</sup> ac-

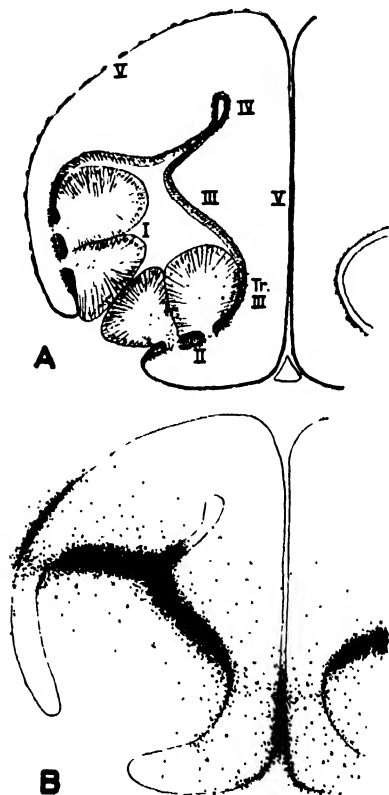


FIGURE 13. Determination of distribution of radioactive iodine within endostyle of lamprey. *A* is a diagram of cell types in a section of an anterior chamber of the endostyle. *B* is the radio autograph made by this section projected on a tracing of the same section. In this way the cell types in which iodine has been accumulated are identified. (From GORBMAN & CREASER.<sup>125</sup>)

curate to 0.005 mg., revealed no iodine.<sup>131</sup> In TABLE 6, additional data are presented. It would appear that the lamprey thyroid contains the active thyroid principle which can be detected by the Gudernatsch tadpole test, but the ammocoete transformation is not affected by those substances known to be potent accelerators in the amphibian.<sup>132-135</sup> It is true that the thyrotrophic factor of the pituitary appeared to bring changes in the endostyle cells,<sup>126, 136</sup> but hypophysectomy was not followed by any detectable change in the endostyle.<sup>136</sup> Iodine and thyroid had no effect upon ammocoete respiration.

In view of these observations, the conclusion of Horton that the thyroid gland has no significance in the metamorphosis of the lamprey still appears to be valid.

*Fish.* The experimental data dealing with the role played by the thy-

Radio Autographs of Sections of  
Developing Thyroids

Length, mm.		Thyroid, 10 $\mu$ section (120 x)	Radio Autograph (120 x)	Radio Autographs of Whole Lower Jaws (Figures denote body length, mm.)  (4 x)
Total	Body			
9	3			
10	3			
11	3.75			
12.5	4.25			
12	5			
(Adjacent sections)				
12	5			
22	8			
24	10			

FIGURE 14. First faint traces of storage of radioactive iodine in thyroid of 10-mm. (total length) tadpole. At this stage each of the two thyroid lobes consisted of 3 to 6 follicles. (From GORRMAN & EVANS,<sup>121</sup>)

roid gland in the physiology of the fish have been negative or contradictory. No one, to my knowledge, has been able to alter the oxygen consumption by the administration of the thyroid substance or thyroxin.<sup>137-</sup>

TABLE 6  
SUMMARY OF EXPERIMENTATION ATTEMPTING TO ACCELERATE  
METAMORPHOSIS IN LAMPREYS

Investigator	Recipient	Treatment	Result
Jensen <sup>132</sup>	Ammocoetes	Inject iodothyrim	---
Rémy <sup>133</sup>	Ammocoetes	Inj. & immersion (26 d.) iodothyrim	—
Horton <sup>131</sup>	Ammocoetes	Iodoform, I, KI, sheep thyroid extract	—
	Frog	Ammocoetes endostyle, frog muscle	—
	Frog	Lamprey, ox thyroid	+
Young & Bellerby <sup>134</sup>	Ammocoetes	Inject anterior pituitary	—
Stokes <sup>135</sup>	Ammocoetes	Thyroxin 1:500,000	—
		Thyroid 1:120,000 1:500,000	—
Knowles <sup>136</sup>	Ammocoetes	Inject thyrotrophic factor	Accel. changes endostyle cells
	Ammocoetes	Hypophysectomy	No change endostyle
Gorbman <sup>136</sup> (unpub.)	Ammocoetes	Inject thyrotrophic factor	Hyperplasia endostyle cells (III)

<sup>139</sup> That the oxygen consumption can be raised was demonstrated by Hasler and Meyer, who caused an increase by the injection of 2-4 dinitrophenol.

Histological studies have revealed a seasonable change in the structure of the thyroid glands of several teleosts.<sup>45, 140, 141</sup> In the eel,<sup>44, 142</sup> the thyroid appears to be involved in the metamorphosis. The fish, *Periophthalmus*, metamorphoses into a partly terrestrial animal, which transformation, in the opinion of Harms,<sup>143</sup> is under the influence of the thyroid. Hoar, in his analysis of the changes in the thyroid gland of the salmon, described a definite positive correlation between periods of intense growth and tissue changes. Young salmon undergo marked changes before entering the sea, and this transformation has been compared with that of the metamorphosis of the eel and amphibian. The early observations of Gudernatsch,<sup>40</sup> that the epithelium of the thyroid decreases in height and the colloid becomes acidophilic with age, have been confirmed in the salmon and interpreted<sup>45</sup> as evidence that the gland is more active in the young fish. Ferguson's<sup>144</sup> observation that the size of the follicle appeared to be proportional to the size of the fish has been corroborated in the salmon and interpreted as an indication that the gland had been active at some previous time.<sup>45</sup>

The administration of mammalian thyroid substance to several species of fish has produced alterations in growth and maturation.<sup>140, 143, 145, 146</sup> These effects have been in the nature of a decreased growth rate with altered body proportions, exophthalmos, precocious and atypical growth of the anal fin. Another group of investigators<sup>139</sup> was unable to produce any change in one of the species, *Lebistes*, by the use of thyroid powder mixed with the food in a 1:1 ratio or by the addition of thyroxin



to the water. In the light of Gudernatsch's<sup>147</sup> observations following thyroid feeding to the rat, the variability of the above results may be due to the differences in dosage.

The goldfish thyroid was found to respond readily to pituitary extracts from frog, chicken and sheep,<sup>148,149</sup> and that of *Fundulus* was activated by sheep pituitary extract.<sup>150</sup> Pituitary extract from the sole affected the goldfish thyroid very slightly, but this was found to be due to a low titer of thyrotrophic factor and not an inability of the target organ to respond.<sup>149</sup> Proof for the presence of an active principle in the teleost and elasmobranch thyroid glands is provided by the acceleration of amphibian metamorphosis following transplants<sup>120, 151, 152</sup> and feeding<sup>205</sup> of these glands. A condition simulating Graves' disease consisting of exophthalmos, muscular weakness, thyroid hyperplasia, and emaciation occurring in trout was overcome by the addition of iodine to the water.<sup>153</sup>

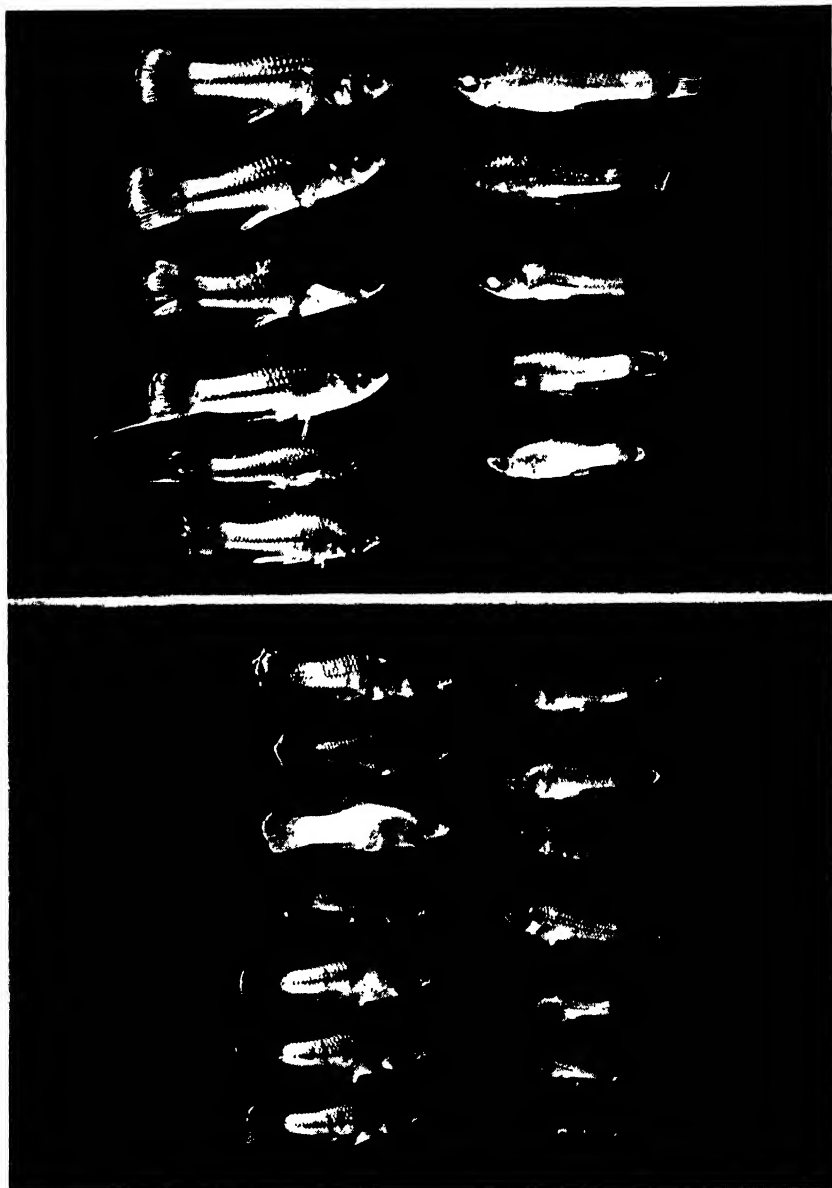
To the best of my knowledge, the critical experiment involving thyroidectomy had not been performed. The isolated follicles and their diffuse distribution make a successful operation extremely difficult. Following the successful use of thiourea and its derivatives as antithyroid agents,<sup>154-166</sup> it occurred to us<sup>167</sup> that chemical thyroidectomy was the answer to a problem of long standing.

In individuals of a *Platyplecillus* × *Xiphophorus* strain immersed in 0.03% solution of thiourea in conditioned water for periods up to 70 days, a retardation of growth, failure of development of secondary sex characters (FIGURES 15 and 16), and a marked hyperplasia and hypertrophy of the thyroid were observed by Goldsmith, Nigrelli, *et al.*<sup>167</sup> The growth retardation was not attributed to inanition, since the treated animals accepted food as readily as did the controls. In another series, of *Lebistes*, Nigrelli, Goldsmith, and Charipper<sup>168</sup> found that small amounts of mammalian thyroid powder added to the diet of the thiourea-treated animals resulted in fish which could not be distinguished from the controls. The inactive thyroids of these animals resembled those of untreated animals, as contrasted with the hyperplastic ones of the fish exposed to the goitrogen. Representative sections of these glands are to be found in FIGURES 17 and 18.

From the combined evidence presented here, it would appear reasonably clear that the thyroid gland functions in the maturation of the fish although it may have no calorigenic function.\*

From this point upward, the story is straightforward. The reviews by Allen,<sup>79</sup> Gudernatsch,<sup>169</sup> Schneider,<sup>87</sup> Gorbman,<sup>170</sup> and Adam<sup>171</sup> make an extensive survey unnecessary. The more recent experimentation utilizing the goitrogens, thiourea, and thiouracil provides additional evidence as to the phylogenetic relationship of the vertebrate thyroid.

\* While this paper was in galley, Smith and Matthews<sup>172</sup> reported that injection of extracts made from the thyroids of Bermuda parrot fish, *Sparisoma* sp., into white grunts, *Bathyloma* sp., resulted in an increase of oxygen consumption in those fish weighing more than 15 grams. Several of the grunts failed to respond and in the light of the weight of negative evidence it would be profitable to repeat the work using greater numbers of fish.



FIGURES 15 and 16. *Platyposyllus* x *Xiphophorus* hybrids immersed in thiourea for 79 and 73 days, respectively. Note the size and differentiation differences between the untreated controls on the left and the thiourea-treated on the right. (After unpublished figure of GOLDSMITH, NIGRELLI, GORDON, & CHARIPPER.)

*Amphibia, Birds, and Mammals.* Goldsmith and his collaborators<sup>157, 164</sup> inhibited metamorphosis of the frog tadpole by immersion in or in-

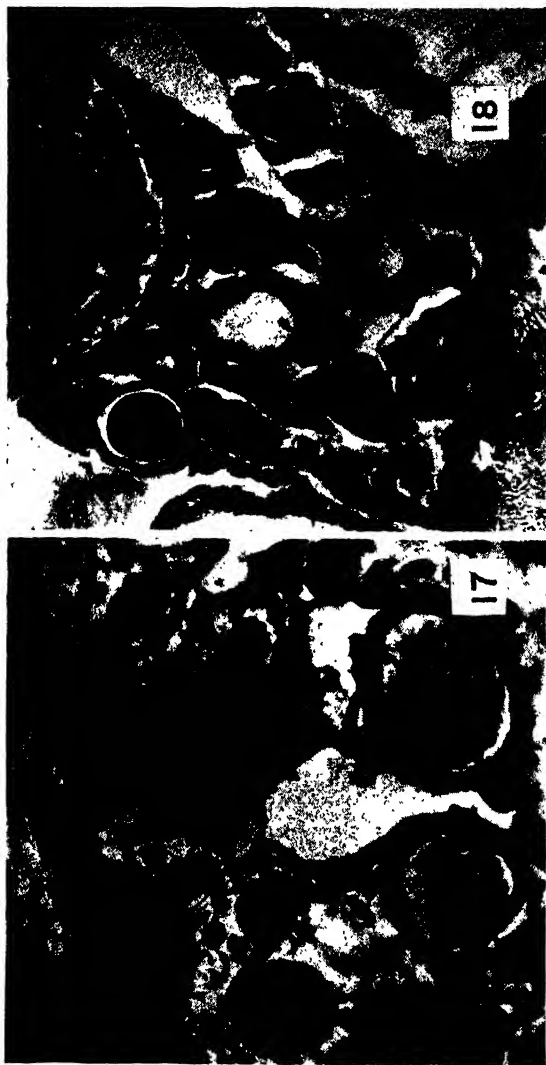


FIGURE 17. Representative section of thyroid gland of untreated *Lebistes reticulatus*. The number of follicles is few and the epithelium is flat. The picture in the fish receiving thiourea-thyroid powder supplement is similar.

FIGURE 18. *Lebistes reticulatus* immersed in thiourea for 4 months. Thyroid gland is hyperplastic, showing abundance of follicles in single field. Widespread proliferation with invasion into branchial region (not shown) is common. Epithelium is much higher than that of control. (Both figures after NIGRELLI, GOLDSMITH, & CHARIPPER, unpublished.)

jection of thiourea. Growth, however, continued with the development of giant tadpoles (FIGURES 19 and 20), reminiscent of those obtained by



FIGURE 19. Note the larger size, arrested development, and crooked tail base condition in the tadpoles immersed in 0.03 per cent thiourea for 58 days since the 20-30 mm. total length stage (lower animals) as compared with normal *Rana pipiens* larvae in several stages of development (top animals).

FIGURE 20. Recently metamorphosed *Rana pipiens* larvae. Animal on the left is a normal frog 104 days old. Animal on the right had been in 0.03 per cent thiourea for 112 days since the 25 mm. total length stage and then returned to water for 102 additional days (chronological age, 232 days). Note the marked difference in size. (After GORDON, GOLDSMITH, & CHARITTER.<sup>161</sup>)

Allen<sup>78</sup> and Hoskins and Hoskins<sup>172</sup> following complete thyroidectomy. Ossification processes were significantly retarded, resembling the effects on the skeleton reported in the thyroidless tadpole.<sup>173</sup> The thyroid picture was one of extreme activation - marked hyperemia, columnar epithelium, scanty colloid. The thiourea effect could be negated by exposure to thyroxine but not by the administration of thyrotrophin. Return of short- and long term thiourea-treated animals to water was followed by a resumption of metamorphosis and a return to the normal histological pattern in the thyroid.<sup>164</sup> Thiouracil has also counteracted the action of the thyrotrophic factor when administered to tadpoles.<sup>161</sup>

Dwarfism in chickens analogous to human myxedema infantilis has been reported by Landauer.<sup>174</sup> These observations, those by Schwarz<sup>175</sup> and many others (see bibliographies<sup>87, 175</sup>) have firmly established the importance of normal function of the thyroid for the normal development of the skeleton and plumage of the fowl. Here, too, investigators seized upon the goitrogens, thiourea and thiouracil, and it soon became

apparent that the chick thyroid was responsive to these chemicals<sup>177, 178, 179</sup> when given in the proper dosage (0.1%) for suitable periods of time (about 14 days).

A wealth of evidence has been accumulated demonstrating that syndromes comparable to myxedema could be produced in many mammals by thyroidectomy. The pertinent facts in the literature may be found summarized in the reviews by Hammett<sup>180</sup> and Schneider.<sup>87</sup> Suffice it to say that thyroidectomy in the immature mammal has long been known to be followed by a retardation of growth—sheep and goats,<sup>181, 182</sup> rabbits,<sup>183</sup> rats.<sup>184-186</sup> More recently, it has been reported<sup>187-189</sup> that surgical removal of the thyroid one to three days after birth results in a marked retardation of growth and maturation in the rat.

By incorporating thiourea in the diet of the pregnant rat, Goldsmith, Gordon, and Charipper<sup>158, 163</sup> thyroidectomized rats prior to birth. Following parturition, the rats received the goitrogen from their mother's milk and, after weaning, directly from the thiourea diet. In a case where the female had been treated throughout the entire gestation period, two scrawny female young were born. Treatment was continued and they developed at a greatly retarded rate, exhibiting definite cretin characteristics. At the age of 84 days, one weighed 50 gm. and the other 78 gm. as compared with 160 gm. for the control females (FIGURE 21).

The thyroid glands of all treated animals appeared enlarged and hy-



FIGURE 21. Thiourea and growth. 84-day-old thiourea-treated animal (50 gm.) as compared with an untreated (160 gm.) of the same age. The cretin was obtained from a mother treated with thiourea during the entire gestation period. (After GOLDSMITH, GORDON, & CHARIPPER.<sup>163</sup>)

peremic, macroscopically; microscopically, irregularity of the follicles, high columnar epithelium, and limited quantities of lightly staining colloid were to be seen. The thyroid of the 50-gm. cretin weighed 50 mg. as compared with a 12 to 15 mg. gland, which is typical for our untreated rats. Somewhat similar results have followed injection of thiouracil in one day-old rats.<sup>190</sup> Several recent papers pertaining to the human<sup>191-193</sup> have confirmed the above findings.

The similarity in results obtained in all classes of vertebrates following the administration of thiourea and thiouracil lead one to adduce that the synthesis of thyroid hormone is similarly inhibited in all of them, as was first demonstrated in the mammal by Keston, Goldsmith, *et al.*<sup>166</sup> and by Chaikoff and his collaborators.<sup>194</sup>

*Reptiles.* Comparatively little has been done with the reptile. What facts there are, however, are of a positive and uniform nature and in agreement with the substantial data which have been amassed for the amphibian and mammal.

The lizard<sup>195</sup> and snake<sup>196</sup> pituitaries have been altered following thyroidectomy, with an increase in the size and number of vacuolated basophiles in the latter. Hypophysectomy in the snake<sup>197, 198</sup> is followed by a change from the cuboidal to the squamous type of epithelium in the thyroid. Administration of anterior pituitary activated both normal (resting)<sup>199</sup> and hypophysectomized snakes.<sup>198</sup> The fence lizard thyroid<sup>149</sup> responded to injections of frog, chicken, and sheep pituitaries. Here, too, the sole pituitary was ineffective. Thyroids of *Anolis* treated with estrogens were found to be more active histologically than those of untreated controls. This was correlated with an accumulation of a colloid-like (gonadotrophic or thyrotrophic hormone) material in the pituitary.<sup>200</sup>

*Thyroid and Tissues in General.* A mass of data has been accumulated<sup>87</sup> in support of the view that thyroid extract or thyroxin applied to isolated vertebrate, invertebrate, and plant tissues accelerated either anabolism, catabolism, or both. That mammals may produce extrathyroidal hormone may be adduced from the observations of a number of investigators.<sup>201-203</sup> It is true, further, that a number of the invertebrates do produce diiodotyrosine, but to the best of our knowledge synthesis of the thyroid hormone takes place for the first time in the adult lamprey gland, and it is in the lamprey that we are able to trace the development of the definitive follicles from endostyle cells.

### *Recapitulation*

Classical developmental studies and experimentation utilizing thyroid and anterior-pituitary preparations, antithyroid agents, and radioactive tracers have resulted in the accumulation of considerable evidence strongly indicating a phylogenetic relationship of the thyroid gland from

the lamprey to man. Further, they support the view that there is a common factor underlying thyroid function in the vertebrates.

Thyroid gland substance, when used in suitable concentrations, appears to influence metabolism in isolated tissues of the vertebrates and in the invertebrate animal.

Below the cyclostomes, where no recognizable thyroid tissue has been described, the possibility of thyroid hormone synthesis remains unanswered.

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### Discussion of the Paper

DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

I should like to comment and elaborate somewhat on some of the interesting points brought out in Dr. Goldsmith's presentation.

(1) In reference to the primary connection of the developing thyroid with the pharynx, as still seen in even the highest vertebrates, including man, there exists a considerable amount of anatomical and embryological data indicating that all glands now of internal secretion have their phylogenetic ancestry in former glands of external secretion, *i.e.*, all glands now "ductless" once had an open secretory duct. The term "thyroglossal duct" is an appropriate anatomical term. Its occasional developmental anomalies (*e.g.*, the persistence of its lumen) as well as certain pathological structures arising from it (*e.g.*, cysts, neoplasms) are well known.

Thus, the thyroid, too, at one stage of vertebrate ancestry existed as a "duct-gland," most likely as a gland auxiliary to the metabolic functions of the alimentary canal, since it poured its secretion into the lumen of the latter. It has retained this metabolic function even after having lost its direct connection with the digestive tract.

An analogous situation existed at one time in reference to other endocrine structures which now are "ductless glands."

(2) All endocrine glands consist of numbers of functional units (isolated cells, clusters of cells, islands, follicles) which have no other connection with each other except by means of the interstitial connective tissue stroma, usually surrounded by a connective tissue capsule. The units are independent of each other functionally, in some glands also anatomically (for instance, chromaffin nodules, pancreatic islands, parathyroid nodules, etc.). Dispersion is a primary characteristic feature of all endocrine structures.

Dr. Goldsmith showed some pictures of the wide dispersion of thyroid follicles in teleost fishes. In some species of the latter, the entire gill region ventrally and laterally to the pharynx is dotted with follicles entirely separate from each other. As he pointed out, there is, in these forms, no connective tissue capsule by which one could anatomically define the "thyroid gland." Structurally speaking, there is no "thyroid gland" in these lower vertebrates, but there are many thyroid follicles.

This is the primary phylogenetic condition, a semblance of which is noticeable during the early embryonic period of all, even the highest

vertebrates. The capsule is a later acquisition. Its late formation explains why accessory thyroid nodules are nothing abnormal and why one often finds "foreign tissues," such as parathyroids, muscle fibers, ganglia, etc., within the confines of the human thyroid.

Dr. Goldsmith also referred to the fundamental studies by Marine on thyroid overgrowth in some teleost fishes (trout) and the influence of iodine in checking and reducing such overgrowth. In view of the dispersive state of the thyroid in these forms, one has difficulty in deciding by what degree of dispersion the normal state changes to the pathological. Unless there is extensive formation and spreading of new follicles (which Marine found to a most impressive degree), one is uncertain when to name the spreading a hyperplasia of the teleost thyroid. Still more hazardous would be the designation "neoplastic" thyroid, unless one finds pathological criteria other than mere increase in number and spreading of follicles.

As shown by Marine, the fish thyroid becomes more dispersed (hyperplastic) under the lack of iodine (combined with some other accidental factors). The reverse, involution, takes place under improved conditions. In the encapsulated thyroid of the human, dispersion of follicles is impossible, but hyperplasia occurs just the same. With the formation of new follicles and the general enlargement of all, this hypertrophy often continues to a very impressive degree (formation of a goiter).

(3) Dr. Goldsmith has given a very logical picture of the endostyle found in the lower vertebrates as being the early phylogenetic structure from which later the thyroid gland may have developed. In general, anatomists and embryologists are agreed on this, though there is a difference of opinion as to whether in all species which possess them the structures called "endostyles" are homologous among themselves and, in turn, comparable to a primary ancestral anlage of the thyroid.

Why does the anatomist make so much of this endostyle-thyroid relationship and why does he search, in general, for the ancestry of endocrine structures further and further down in the phylogenetic scale, now even in the invertebrates? He is looking for the continuity of these organs throughout the entire phylogenetic tree. In most instances, this anatomical continuity is well established, especially for the thyroid. But even if it were not, even if our present interpretation of the endostyle were incorrect, we have a well-established chemical (which means physiological) endocrine continuity throughout the vertebrates—as regards "thyroid chemistry" even throughout the invertebrates. (Sugar-reducing substances, estrogen-like and other compounds resembling the hormones of higher forms, have also been found in invertebrates. Their specific function there, if any, is not known.)

Iodine is present in all invertebrates examined. Naturally, it would be present in all marine forms. Specific iodine-amino acid combinations, likewise, are present in invertebrates. One of the essential constituents of the hormonal complex of the thyroid, 3,5-diiodotyrosine, was first iso-

lated from one of the lowest invertebrate forms, *viz.*, some species of the Gorgoniidae (corals such as those used for jewelry). It was then named iodogorgonic acid (Drechsel). Around 1905, Lafayette B. Mendel isolated 3,5-diiodotyrosine from Florida sponges, the lowest forms of invertebrates just above the Protozoa. Thus, the point emphasized by Dr. Goldsmith that there is phylogenetic continuity as regards the "thyroid complex" from the lower forms up is well taken. Even though we may not be able to uncover morphological evidences of such continuity, we find chemical continuity throughout, some iodine always being attached to some protein constituents. How far down in the phylogenetic scale such forerunners of thyroid hormonal compounds exert a physiological action resembling that of the true hormone in the higher forms, is unknown. Dr. Goldsmith emphasized correctly the "physiological interchangeability" of hormones across the species borders, meaning that certain hormones obtained from one species, high or low, will produce their principal effects in another species, no matter how high or low (endocrine therapy in man very often makes use of hormonal substances obtained from other forms).

(4) Dr. Goldsmith discussed the influence of thyroid material (mammalian material effective in amphibians) in the accelerated metamorphosis of tadpoles and stated that this phenomenon demonstrates the specific influence of the thyroid on the maturation process of the juvenile organism. During metamorphosis, the immature tadpole (the amphibian "paedoform") progresses toward the mature form, the permanent adult frog. The concomitant anatomical changes are very drastic in these lower animals, especially when hastened by the administration of thyroid material. At the same time, pronounced changes in the physiology of these forms take place.

In the human, too, we observe a series of such anatomical and functional changes when the organism progresses from the paedo- to the mature form. These changes, occurring in man during the period of adolescence, are more gradual than those in lower forms where the body changes over completely from one anatomical type to another; yet, they are "metamorphic" changes just the same (body configuration, skeletal changes, secondary sex characters, etc.). As in the advance from tadpole to frog, in the human, too, these changes are brought on, and in retarded cases accelerated, by hormonal factors, a properly functioning thyroid being their prerequisite. That this is the case may be judged from the study of "subnormal" adolescent individuals. If unaided, an athyroid individual will remain permanently infantile (in fact, even below the normal paedoform stage), just as an athyroid tadpole will never change into a frog. Hypothyroid individuals will develop to a state of "maturity" commensurate with the degree of their hypothyroidism. By thyroid medication (usually coupled with pituitary and gonadal hormones), the physician succeeds in bringing such individuals forward to a more advanced stage of maturity, even though they may not be able to reach the func-

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tional adult stage. The earlier in life such treatment is initiated, the better the chances for advancement, showing clearly that the thyroid exerts its *maturing* influence during the *entire* period of development, and that it is capable of accelerating the rate of development very early. A tadpole can be changed by thyroid application into a frog at the age of 15 days, while normally it would metamorphose at 75 to 80 days.

In adolescent humans who fail to go through the final steps to full maturity because of lack of thyroid drive, maturation can easily be accelerated and completed by thyroid medication. Closure of various epiphyses, growth of facial and pubic hair, development of breasts, onset of menstruation, descent of testicles in cryptorchids when there is no anatomical impediment in the inguinal canal, are signs of this transformation and maturation.

It is appropriate, therefore, as Dr. Goldsmith has emphasized, to consider the thyroid hormone as a *maturation hormone*, at least in the particular phase of thyroid function here discussed.

# CYTOLOGICAL AND CYTOCHEMICAL BASES OF THYROID FUNCTION\*

By F. DE ROBERTIS†

*Massachusetts Institute of Technology, Cambridge, Massachusetts*

THE thyroid gland is the only one among the endocrine system which, at the same time that it synthesizes its secretion, stores it in great quantities outside the cells in follicular cavities. This double function of synthesis and storage determines the architecture of the organ, whose anatomical unity is the thyroid follicle.

For these reasons, any study of the cytological and cytochemical foundations on which thyroid function might be based is more complex than in other endocrine glands. Here, one has to consider not only the processes by which the hormone is synthesized, secreted, and stored, but also the mechanisms by which this hormone is reabsorbed and enters into the circulation to be utilized by the organism. As the present Conference demonstrates, this study has progressed a great deal in the last few years, due to the introduction of many new methods of research belonging to the various fields of anatomy, physiology, biochemistry, and biophysics. In this paper, I shall refer particularly to results of the use of cytological and histochemical methods, and especially to the work carried on by us since 1941 at the University of Buenos Aires.

*The Intracellular Colloid.* Cytological methods have contributed a great deal to the knowledge of the function of endocrine organs but in many cases have failed to show the intracellular secretory processes. Sometimes this is due to the lack of appropriate or specific methods for detecting the products of secretion. In other cases, processes may occur exclusively in the submicroscopic level and the biochemical phenomena are not revealed by visible changes under the microscope.

In the case of the thyroid gland, the literature from 1888 until 1939 reveals great confusion, not only concerning the elements which compose the intracellular colloid secretion (colloid droplets of Biondi, chromophobic vacuoles of Anderson, granules of Galeotti, of Lobenhofer, of Kinoshita, basal colloid of Bensley, granules stainable by the neutral red of Uhlenhuth, etc.), but also on the possibility of their actual identification. When we started our work on the subject, the most recent reviews on the histophysiology of the thyroid gland, like those by Ponse (1938), Cowdry (1938), Bloom (1939), and Bargmann (1939), had reached the conclusion that secretion of the thyroid gland is invisible or that the intracellular secretory processes could not be observed microscopically with certainty.

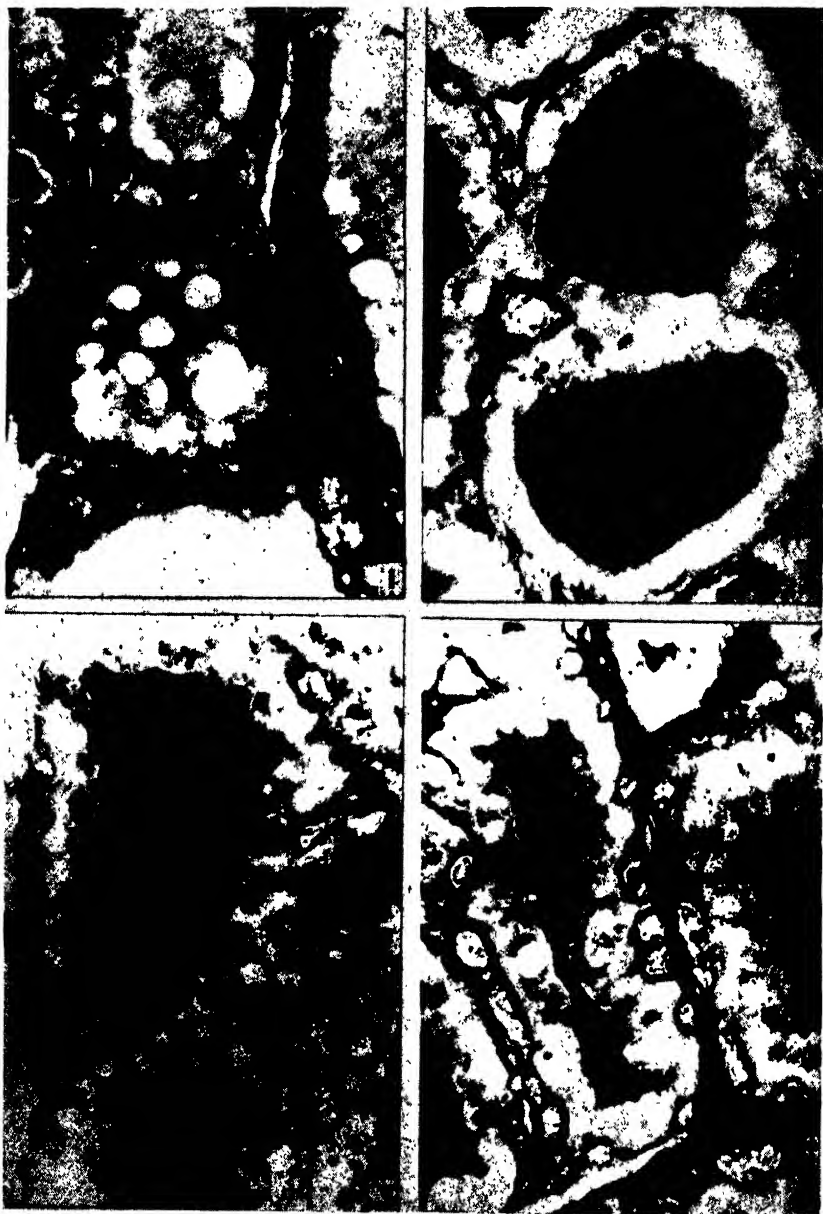
\* Work done mainly at the Institute of General Anatomy and Embryology, University of Buenos Aires, Argentina.

† Fellow of the Guggenheim Foundation.

*A priori*, it might be thought that this uncertainty was due to the lack of adequate technical methods for fixing and staining the colloid within the cell and, for this reason, in 1940, we used the freezing-drying method of Altmann-Gersh. This preserves best the different cellular components, particularly the proteins taking part in the secretion. This technique, followed by a simple staining method, reveals the products of secretion and reabsorption inside the cells, which we designated as intracellular colloid (De Robertis, 1941a), and we undertook a study of its changes in different physiological and pathological conditions. FIGURES 1 and 2 show the difference between the image produced by a chemical fixative like Regaud's fluid (FIGURE 1) and the freezing-drying technique (FIGURE 2). In the first figure, the follicular colloid shows many clear vacuoles, situated mainly at the periphery of the follicles, as is usual with common techniques. These vacuoles, described by all the authors since Anderson (1894), have been interpreted by some as chromophobic colloid being secreted (Anderson, 1894; Uhlenhuth, 1927; Charipper, 1926; etc.) and by others as vacuoles of reabsorption (Aron, 1930; Severinghaus, 1933) because they appear especially in activated glands. However, recently, several investigators have indicated that those vacuoles could be interpreted as artifacts, basing their supposition mainly on the action of different fixatives (Bucher, 1938) or on observations on living glands (Hartoch, 1933; Vonwiller and Vigodskaya, 1934; Williams, 1937-40; De Robertis, 1941b).

The freezing-drying method which was also applied by Gersh and Caspersson (1940) for observations with ultraviolet light showed definitively, in our hands (De Robertis, 1941a), that follicular vacuoles are artifacts produced by the fixatives while precipitating the gel which fills the thyroid follicle. Also, in strongly activated glands the follicular colloid is homogeneous (FIGURES 3 and 4).

Comparing both figures (1 and 2), it is seen also that, in the second case, the cells show the presence of colloid droplets inside the cytoplasm. The amount and disposition of the intracellular colloid varies with the species, with age, and with the season, and most markedly with functional (FIGURES 3 and 4) or pathological conditions in the gland. The colloid generally appears as droplets situated in the apical zone of the cell, but more rarely it is found in the basal part of the cell as large, pale-stained droplets similar to those described by Bensley (1916) as basal colloid. In a few cases, particularly in hyperactive glands, all the colloid droplets of a cell are at the base and the cell shows a complete inversion of polarity (FIGURE 6; De Robertis, 1941c). That the intracellular colloid described by us contains the thyroid hormone, seems to be apparent from a comparison of our results with those obtained by Gersh and Caspersson (1940) using the microspectrographic technique in ultraviolet light, with which they found specific absorption curves of organic iodine in vacuoles situated in the apical part of activated cells. We compared the photomicrographs obtained by the authors with the slides from the



FIGURES 1 to 4, photomicrographs of thyroid glands taken with 3 mm. objective and 12 X ocular.

FIGURE 1. Normal thyroid of a young rat. Regaud's fixation and Bensley's acid fuchsin-methyl green technique for mitochondria. Intrafollicular colloid shows many artificial vacuoles.

FIGURE 2. Normal thyroid of a young rat. Frozen-dried denatured stained with aniline blue-orange. Cuboidal epithelium with few colloid droplets. Homogeneous colloid.

FIGURE 3. Rat of the same litter three hours after the injection of TSH. Cells are higher and show many colloid droplets. Colloid is being released.

FIGURE 4. Rat of the same litter ten days after injections of TSH. High cylindrical cells with many colloid droplets. Colloid greatly released.

same material (given to us by Dr. Gersh) made with our staining technique. However, more direct evidence is lacking.

From these results, it is also clear that the chromophobic secretion considered by many authors, such as Anderson (1894), Uhlenhuth (1927), Baillif (1937), von Hagen (1938), etc., to be the true thyroid secretion, probably is an artifact or is due to an incapacity of common fixatives to preserve the content of those vacuoles.

When rats or guinea pigs are injected with TSH, within 15 minutes one can find a great increase in intracellular colloid. Analysis of this early activation in rats and guinea pigs shows (De Robertis, 1942, and unpublished results; FIGURE 5) that, in an initial period (with its maxi-

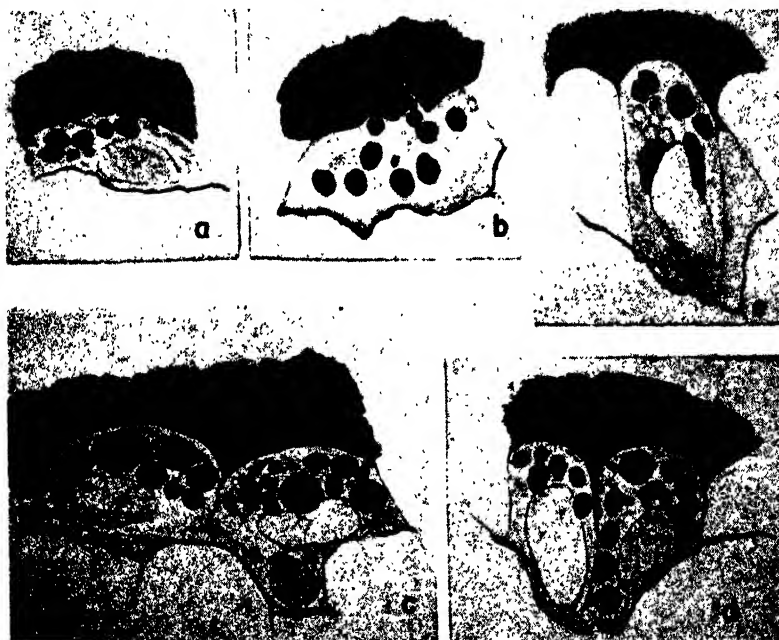


FIGURE 5. Drawings made with the help of the camera lucida of frozen-dried denatured sections of thyroid glands, stained with aniline blue-orange. 2150 X.

a, b. Thyroid cells of guinea pig 30 minutes after the injection of TSH. Phase of secretion toward the lumen. In a, a colloid droplet still surrounded by a rim of cytoplasm is seen in the lumen.

c. Phase of release six hours after the injection of TSH.

d, e. Phase of release six hours after the injection of TSH. Cells are higher, with convex apical border. Clear basal droplets of dilute colloid are seen.

mum at 60 minutes), the cells are actively secreting toward the lumen. Colloid droplets which are formed near the nucleus move toward the apex, at the same time increasing in size, and are excreted into the follicular cavity, FIGURE 5a, b. It may be that the excretion of these droplets into the lumen is preceded by a diminution of the surface tension in some parts of the apical surface. At any rate, at these points the cytoplasm enclosing the colloid droplets bulges into the lumen, to be released

finally by rupture of the pedicle. These processes suggest that thyroid cells produce an apocrine secretion, parts of their cytoplasm being excreted together with the secretion into the lumen.

After this phase, the cells appear to stop secreting toward the lumen, and to begin secretion toward the base, reabsorbing the colloid stored in the lumen. This assumption is further supported by the observations that (1) pale-staining, seemingly dilute colloid appears in the base of the cells; (2) in some thyroid cells, small colloid droplets are distributed in the basal half, resulting in a tendency toward a complete inversion of morphological polarity (FIGURE 6); and (3) intrafollicular colloid diminishes.

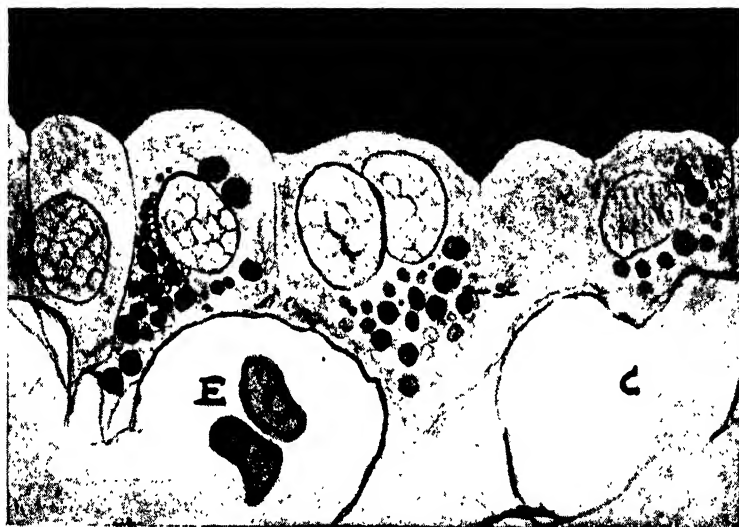


FIGURE 6. Thyroid cells of a rat injected 22 hours before with TSH. Inversion in the polarity of colloid droplets is shown. *ci*, intrafollicular colloid; *sc*, intracellular colloid; *E*, erythrocyte; *c*, capillary.

In the amphibian, the first phase of secretion toward the follicle could not be observed and a reabsorption with great increase of intracellular colloid took place (De Robertis and Del Conte, 1942).

*The Cytological Method for the Assay of Thyrotropic Hormone.* After the first experiments on rats, it became clear that the changes in amount and disposition of intracellular colloid were a very sensitive index of the functional activity of the gland and that these changes reflected in a sensitive manner the amount of circulating thyrotropic hormone. One species considered as insensitive to the TSH (Aron, 1932; Houssay *et al.*, 1932; Thurston, 1933), namely, the rat, reacted with a definite increase in intracellular colloid with 0.5 Junkmann-Schoeller unit (De Robertis, 1942). More recently, there was found a definite reaction with 0.1 (Grasso, 1946) and even with 0.01 of a unit (unpublished results). In a sensitive animal like the guinea pig, we found a definite increase of thyro-

tropic hormone with 0.001 and even with 0.0002 of a unit. Furthermore, the reaction seemed proportional to the dose so that a quantitative method was developed based on the relative amount of intracellular colloid (De Robertis and Del Conte, 1944). The technique consists in injecting the sample intravenously into the guinea pig, and in fixing the gland in liquid air 30 minutes later. Afterwards, the tissue is dried *in vacuo*, denatured and stained as usual, and the number of colloid droplets from 10 follicles is measured. A cytological coefficient was established as the ratio between the number of colloid droplets times 100 and the average diameter of the follicles:

$$Cc = \frac{\text{No. of droplets} \times 100}{\text{Medium follicular diameter}}$$

The cytological coefficient of the normal guinea pig thyroid is about 9.4, while in the rat it is a little higher, 13.04 (Grasso, 1946). As seen in FIGURE 7, the cytological coefficient increases with the dose and there

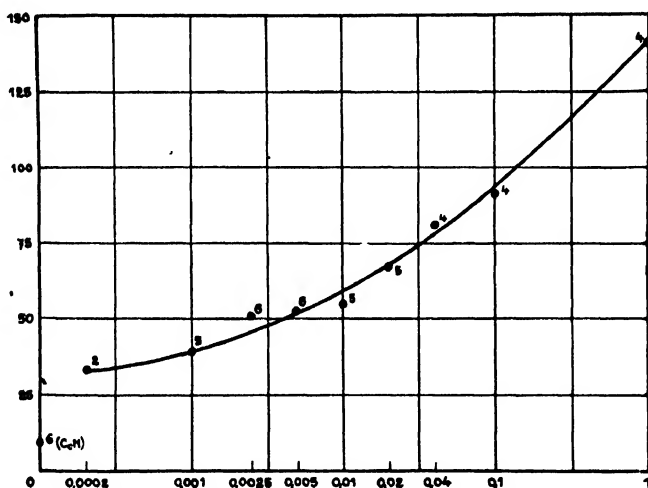


FIGURE 7. Curve plotting the relation between the cytological coefficient (0 to 150) and the doses of TSH 90.0002 to 1 Junkmann-Schoeller unit injected into the guinea pig expressed in logarithmic scale. Points indicate the means of the results for each dose and the number of animals. *CcN*, normal coefficient.

exists an approximate proportion between this coefficient and the logarithm of the dose. Direct methods for the assay of TSH are generally based on changes in the weight of the thyroid gland and especially on histological changes such as the increase in height of thyroid cells. (For a discussion of the literature, see De Robertis and Del Conte, 1944.) This particular method is based on a cytological variation in the amount of intracellular colloid, and we find these changes highly specific. This cytological method greatly surpasses in sensitivity other techniques for the assay of TSH. In fact, Rawson and Salter (1940), using changes in

cell height in day-old chicks, could demonstrate only 1/8 of a J-S unit injected during 5 days. Galli-Mainini (1943), in the hypophysectomized toad, found the maximum of sensitivity with 1/10 of a unit. The direct method of D'Angelo Gordon and Charipper (1942) on frog tadpoles permits the determination of 1/320 of a unit, or, when indirect phenomena such as changes in metamorphosis (which are not completely specific) are used, a sensitivity of 1/1,280 of a unit can be attained. However, a great limitation for the use of this method is the small amount of fluid that can be injected into the tadpole (0.05 ml.), so that the TSH can be determined only in liquids where it is in rather high concentrations (1/16 unit per ml.).

Using the cytological method, if one injects 1 or 2 ml. of fluid into a guinea pig, one can demonstrate TSH in a concentration of 1/5,000 or 1/10,000 of a unit per ml. With this method, it is possible to demonstrate the presence of TSH in 2 ml. of normal human blood and to find great changes in amount with different pathological conditions. We believe that the specificity and extreme sensitivity of this method, when applied to human fluids, may permit clarification of the exact role of TSH in the pathology of the thyroid gland.\*

*The Oxidation-Reduction Potential and the Peroxidase System of the Thyroid Follicles.* In 1941, we found that the pH of the follicular colloid determined by a microdissection technique did not change with the state of activity of the gland. We then became interested in the histochemical study of the oxidation-reduction potential as an expression of thyroid physiology. This interested us because, in a given cell or tissue, the level of the oxidation-reduction potential is determined by the activity of the enzymatic systems present, the  $O_2$  pressure, and the nature and concentrations of the substrates (Stiehler and Flexner, 1938). It may also indicate how the oxidation-reduction energy can be utilized in the secretory mechanism. Even more interest arose when it was found that a series of substances such as thioureas and sulfonamides inhibit thyroid secretion, opening in this way a new field in thyroid physiology and a better approach to the study of the mechanism of the synthesis of the thyroid hormone.

The oxidation-reduction potential of the follicular colloid was determined in the thyroid of the rat by introducing various indicators into the lumen with a micropipette or by extracting the colloid from a single follicle and mixing it in the micropipette under the microscope with a small amount (about  $2 \times 10^{-6}$  ml.) of the indicator. The potential of the thyroid cells and of the colloid was studied by the supravital technique described by Friedenwald and Stiehler (1938; see De Robertis and Moura Gonçalves, 1945). Results are shown in TABLES 1 and 2. It is seen that, in the normal rat, the potential of the cells is somewhere between +0.110 volts and +0.047 volts, while in the colloid it is between -0.167 and

\* Data on the assay of TSH in blood of human patients will soon be published. (DE ROBERTIS, E. P. 1948. J. Clin. Endocrinol.)



TABLE 1

## OXIDO-REDUCTION POTENTIAL OF THE COLLOID DETERMINED BY MICRODISSECTION

<i>Indicators</i>	<i>Eo' at pH 7</i>	<i>Normal rats</i>	<i>Activated rats</i>	<i>Action KCN in activated rats</i>	<i>Action thiourea in activated rats</i>
2-6 Dichlorophenol-indophenol	+0,217		+		
Thionin (Lauth's violet)	+0,100*	+	+		
Cresyl blue	+0,047	+	—		
Methylene blue	+0,011	+	—	+	+
Indig. tetrasulfonate	-0,046	+	—	+	+
Indig. trisulfonate	-0,081	+			
Nile blue	+0,122	+		+	+
Cresyl violet	0,167	+	—	+	+
Phenosaphranine	-0,252	—		—	—
Neutral red	-0,330	—		—	—

\* Eo' at pH 6.0.

TABLE 2

## OXIDO-REDUCTION POTENTIAL OF THE COLLOID DETERMINED BY SUPRAVITAL TECHNIQUE

<i>Indicators</i>	<i>Eo' at pH 7</i>	<i>Normal rats</i>		<i>Activ. rats</i>		<i>Action KCN</i>		<i>Thiourea</i>		<i>Sulf.</i>	
		<i>Cells</i>	<i>Colloid</i>	<i>Cells</i>	<i>Colloid</i>	<i>Cells</i>	<i>Colloid</i>	<i>Cells</i>	<i>Colloid</i>	<i>C.</i>	<i>C.</i>
Thionin (Lauth's violet)	-0,110*	+	+	+	+	+	+	+	+	+	+
Cresyl blue	+0,047	—	+	—	—	+	+	+	+	—	+
Methylene blue	+0,011	—	+	—	—	+	+	+	+	—	+
Nile blue	+0,122	—	+	—	—	+	+	+	+	—	+
Cresyl violet	-0,167	—	+	—	—	+	+	+	+	—	+
Phenosaphranine	-0,252	—	—	—	—	—	—	—	—	—	—
Neutral red	-0,330	—	—	—	—	—	—	—	—	—	—

\* Eo' at pH 5.8.

—0.252 volts. When the gland is activated by the action of cold or by the injection of TSH, the potential of the colloid rises to the same level as that in the cells. Thiourea and KCN lower the potential of the cells and also that of the colloid in the case of activated glands. However, sulfonamide, another inhibitor of thyroid function, did not change the potential. These results led us to investigate the enzymatic systems that could be involved, particularly the oxidative systems. While this work was under way, there appeared the finding by Dempsey (1944) of the presence in the thyroid cells of a peroxidase activity which was inhibited by thiouracil. This observation was particularly important because peroxidases catalyze the liberation of iodine from iodides and their presence in the cells may be related to biological iodinations (Keston, 1944) and to the formation of thyroxine (Westerfeld and Lowe, 1942). The peroxidase activity was, therefore, studied with different techniques and in different conditions (De Robertis and Grasso, 1946). The results are very briefly summarized here. In the colloid extracted by microdissection (TABLE 3), both indophenoloxidase and peroxidase were negative in normal conditions, but the last was positive in glands activated by cold or by TSH. Both thiourea and KCN in concentration M 0.005 inhibited the reaction of the activated glands. With a supravital

TABLE 4  
PEROXIDASE ACTIVITY OF CELLS AND COLLOID, DETERMINED WITH THE SUPRAVITAL TECHNIQUE

Peroxidase Eo' at pH 7 (volts, approx.)	Normal		Activated		Action of KCN		Action of thiourea		Action of sulfanilamide	
	Cells	Colloid	Cells	Colloid	Cells	Colloid	Cells	Colloid	Cells	Colloid
±0.050	+	—	+	+	—	—	—	—	+	—
—0.200	±0.050	—0.200	+0.050	±0.050	—0.200	—0.200	—0.200	—0.200	+0.050	—0.200

TABLE 3

INDOPHENOLOXIDASE AND PEROXIDASE ACTIVITY IN THE COLLOID  
EXTRACTED BY MICRODISSECTION

	<i>Normal rats</i>	<i>Activated by cold</i>	<i>Activated by TSH</i>	<i>Action of thiourea on activated glands</i>	<i>Action of KCN on activated glands</i>
Indophenoloxidase	—	—	—		
Peroxidase	—	+	+	—	—
Eo' at pH 7 (approx., volts)	0.200	0.050	-0.050	-0.200	-0.200

—, negative; +, positive. Eo', oxidation-reduction potential. TSH, thyrotropic hormone.

technique, the same results were found for the colloid (TABLE 4). In normal and activated cells, peroxidase activity is positive and the reaction is inhibited by KCN and thiourea. However, sulfanilamide had no influence on the peroxidase activity. In both TABLES 3 and 4, results are correlated with the previous determinations of the oxidation-reduction potential.

In TABLE 5, it is shown that the histochemical reaction of peroxidase

TABLE 5

## ACTION OF THIOUREA AND SULFANILAMIDE IN FROZEN SECTIONS

	<i>M 0.01</i>	<i>M 0.005</i>	<i>M 0.0025</i>	<i>M 0.002</i>	<i>M 0.001</i>	<i>M 0.0001</i>
Control ++						
Thiourea		—	—	—	+	++
Sulfanilamide	+++	+++				

is completely inhibited with a concentration of thiourea of M 0.002, while with M 0.001 it is still very weak.

In another series of experiments, the action of thiourea on the liberation of iodine from iodides was tested. As seen in TABLE 6, the reaction

TABLE 6

## ACTION OF THIOUREA ON THE LIBERATION OF IODINE FROM IODIDE

	<i>Control</i>	<i>M 0.03</i>	<i>M 0.01</i>	<i>M 0.005</i>	<i>M 0.0025</i>	<i>M 0.002</i>	<i>M 0.001</i>	<i>M 0.0001</i>
Thyroid extract	+		—	—	—	—	—	+
<i>Id.</i> heated at 120°C.	+		+	+	+	+	+	+
Potato extract	+		—	—	—	—	±	+
<i>Id.</i> heated at 120°C.	+		+	+	+	+	+	+
Hemoglobin	+	—	+	+	+	+	+	+
<i>Id.</i> heated	+		+	+	+	+	+	+
Brain extract	+		+	+	+	+	+	+
Muscle extract	+		—	+	+	+	+	+

was inhibited with M 0.001 thiourea. The same results were obtained with a potato extract containing a vegetable peroxidase. Opposite results were found after heating of the thyroid and potato extract or when using hemoglobin, brain and muscle extracts, which do not contain true

peroxidase. Sulfathiazole also seems to stop the liberation of iodine, but probably by the formation of a new compound.

Time does not permit a general discussion of the literature on the mechanism of action of thyroid activators and inhibitors. We only want to point out that these results lead us to the conclusion that thiouracil acts by inhibiting the peroxidase system, which in turn takes part in the liberation of iodine from iodides. Sulfonamides do not inhibit the peroxidase activity, but probably have a competitive action by which the liberated iodine combines with the sulfa drugs instead of with tyrosine.

(For more details in the discussion of this problem, see De Robertis and Grasso, 1946.)

*The Proteolytic System and the Release of the Colloid from the Thyroid Gland.* The fundamental problem of the release of the colloid stored in the follicles is that of the mechanism by which thyroglobulin is taken by the thyroid epithelium and transferred to the blood and lymphatic capillaries. Such a mechanism could be understood readily on the basis of the laws of permeability if very small molecules were involved. As Chambers and Zweifach (1946) state, "the upper limit of porosity of the cell membrane has never been claimed to be in excess of 342, the molecular volume of saccharose," and Wilbrant (1946) considers a molecular volume of 68,000 as the limiting porosity of the capillaries in the glomerular tufts of the kidney, where the transfer is probably through the cementing substance.

With the ultracentrifugation, it has been shown that thyroglobulin has a molecular volume of 675,000 (Heidelberger and Pedersen, 1936). It is really a macromolecule which, as such, cannot pass through the membranes of the cells.

In 1936, Salter and Lerman, by using total thyroid extracts, were able to synthesize iodoproteins which possessed activity similar to thyroglobulin and suggested the presence of an enzymatic mechanism which could act not only in the synthesis but also in the destruction of the thyroid protein. As Means (1937) stated, "The secretory activity of the thyroid gland could be compared with a reversible chemical reaction obeying the 'law of masses,' " so that, when the predominating reaction is in the direction of the synthesis, there would be an increase in the thyroid-protein (colloid), while when the process is reversed, thyroid-protein would be fragmented and more hormone would be released into the circulation.

In 1940, Gersh and Caspersson suggested the possibility that the follicular colloid could be digested by enzymatic action and the products absorbed by the cells. The same year (De Robertis, 1941b), we tried to confirm this hypothesis by testing the presence of proteases in the follicular colloid extracted by microdissection from single follicles. With this method, we were able to demonstrate the presence, in the colloid, of a proteolytic activity which varies in intensity according to the pH

of the medium and the physiological activity of the gland. It was found that TSH, which produces hyperactivity and reabsorption of the colloid, increases the proteolytic activity, while iodine administered for a long period of time decreases it.

These facts led us to postulate the theory of the enzymatic reabsorption of the colloid, according to which the release of the secretion stored into the follicles takes place only after hydrolysis of thyroglobulin inside the follicular cavity. In the diagram (FIGURE 8), the mechanism is ex-

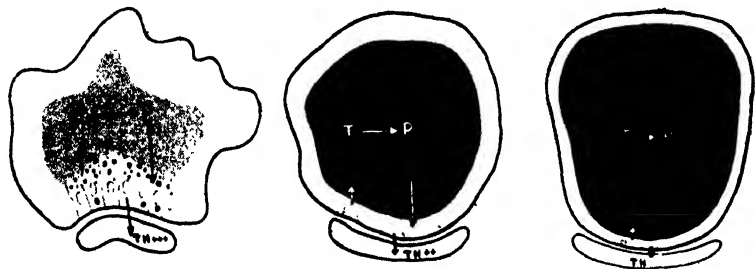


FIGURE 8. Diagram of the enzymatic mechanism of reabsorption of the colloid in normal conditions (middle), in a hyperactive follicle (left), and in a hypoactive one (left). Length and directions of the arrows indicate the intensity of processes of hydrolysis, reabsorption, apical and basal secretion. *T*, thyroglobulin; *P*, product of the hydrolysis of *T*; *TH*, circulating thyroid hormone. (The number of + indicates the amount of *TH* in each case.)

pressed graphically as it occurs in normal conditions and in the case of hyperfunction and hypofunction of the gland.

The experimental facts supporting this theory were fully confirmed by Dziemian (1943), using micromethods for proteases applied to the total gland. The author also found a lower P.A. after hypophysectomy.

These results strongly supported the assumption that a similar mechanism could exist in the human gland and that changes in this enzymatic activity might be related to diseases of the thyroid. In fact, the physiopathological mechanisms involved are essentially similar to those which occur in physiological or experimental conditions.

In toxic goiter, there is an increase in the release of the colloid, called by Holst (1927) and Harington (1933) "leakage" or "diarrhea" of the gland. This causes depletion of the colloidal stores. In simple goiter (at least in the phase of colloid goiter of Marine), there is an opposite phenomenon, namely, a diminution in the release, and a storage of colloid.

For these reasons, we carried out estimations of the enzymatic activity in a series of human thyroid glands representing normal and various pathological conditions (De Robertis and Nowinski, 1946a). It was found that, in cases of severe toxic goiters, there is an increase of P.A. to a point about 100 per cent above normal, while in simple colloid goiters the P.A. is about 30 per cent below the normal level (FIGURE 9). These results seem to indicate that the proteolytic system has an important role in the physiopathology of thyrotoxicosis and simple colloid goiter.

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In cases of mild toxic goiters (starting with a B.M.R. of  $-40$  per cent) which reached a complete relief of the symptoms with the iodine treatment, the P.A. is not only lower than that of severe toxic goiters but is even below the normal level ( $-26.2$  per cent). It is interesting that, in these cases, the gland showed big follicles filled with colloid, as in simple goiter.

This fact, together with the diminution of P.A. found by us (De Robertis, 1941b) and by Dziemian (1943) in rats treated with iodide, may

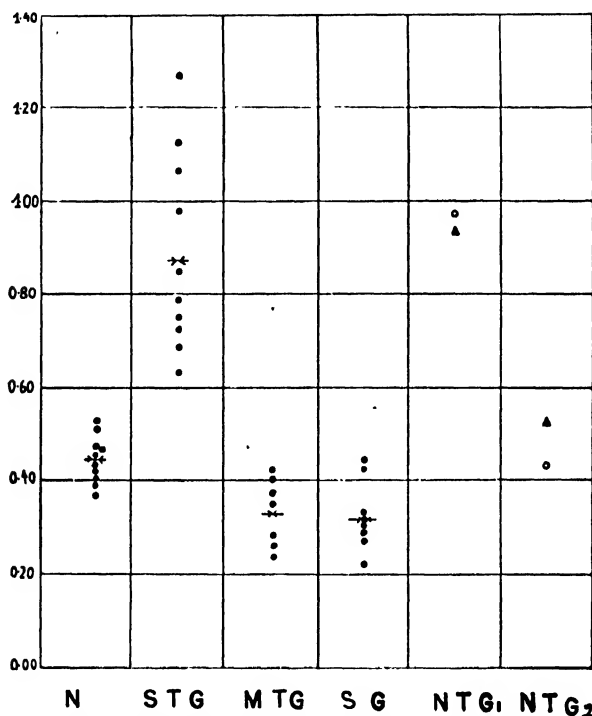


FIGURE 9. Diagram expressing the proteolytic activity of human thyroid tissue in normal glands (N), severe toxic goiter (S.T.G.), mild toxic goiter (M.T.G.), simple goiter (S.G.), nodular toxic goiter, toxic adenoma (N.T.G.<sub>1</sub>), and the rest of the gland (N.T.G.<sub>2</sub>). Proteolysis is measured by the mg. of tyrosine and tryptophane set free during the digestion of edestin by 100 mg. of tissue;  $\rightarrow \leftarrow$  means.

be interpreted on the basis of the enzymatic reabsorption of the colloid and suggest that the action of iodine in thyrotoxicosis may be attributed to the influence on the proteolytic enzyme system of the thyroid gland. The mechanism of iodine action in this inhibition may possibly be due to a direct iodination of the proteolytic enzyme. Support of this idea is found in the paper by Herriott (1936) who, by iodinating pepsin *in vitro*, observed a progressive inhibition in P.A. due to the fixation of iodine in the tyrosine molecule.

In order to check the direct action of iodine upon the proteolytic enzyme of the thyroid, we carried out a series of experiments in which the

glycerol extracts of the gland were iodinated *in vitro* with Herriott's method. We found a strong inhibition of the P.A. (De Robertis and Nowinski, 1946b). However, our interpretation of the mechanism of the therapeutic action of iodine does not exclude the existence of other processes such as the action on TSH recently supported by Rawson and colleagues (1945). It is obvious that, in the case of a process so important physiologically as the regulation of hormone production, the cooperation of several systems must be involved.

### *Summary*

Our knowledge of secretory processes in the thyroid gland was rather confused until 1939 because of the lack of adequate methods for preserving the products of secretion and reabsorption inside the cells. In 1940, the use of the freezing-drying technique enabled us to demonstrate the intracellular colloid. It was also found that vacuoles in the colloid and the so-called chromophobic secretion were artifacts.

Activation of the gland by TSH produces a great increase of intracellular colloid. This is noticeable as early as fifteen minutes after administration of the hormone. At first, the cells secrete toward the follicle, producing a kind of apocrine secretion which is very evident when the cells are strongly activated. Afterwards, apical secretion stops and release of the colloid through the cells is observed. In some cases, there is also an inversion of cell polarity. This increase of intracellular colloid is the most sensitive method for detecting TSH. In the guinea pig, the injection of 0.0002 unit of TSH produces a definite increase of the number of colloid droplets and the reaction is proportional to the dose, at least up to one unit of TSH. A cytological coefficient was established, based on the number of colloid droplets per follicle, and on this basis a method for the assay of TSH was developed.

This method is much more sensitive than others utilized for the assay of TSH, and permits one to demonstrate its presence in 2 ml. of human blood. Thus, it can be widely applied to determine the role of this hormone in different pathological conditions. The study of the oxido-reduction potential of the thyroid follicle shows that it is intimately related to the activity of the gland. In normal glands, the  $E_o'$  of the cells is about  $+0.050$  volts and the colloid  $-0.200$  volts. During activation, the  $E_o'$  of the colloid increases to  $+0.050$ . KNC and thiourea lower the  $E_o'$  of cells and colloid to  $-0.200$ , but sulfa drugs do not change the potential. These results were related to changes in peroxidase activity under similar conditions. The conclusion is drawn that thiourea probably acts by inhibiting the peroxidase system which takes part in the liberation of iodine from iodides, while sulfonamides do not inhibit peroxidase activity and probably have a competitive action toward the essential metabolite.

The release of the colloid through the cells cannot be explained by laws of permeability because of the large size of the thyroglobulin

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molecule. Hence, it was suggested that an enzymatic mechanism might be involved. In 1941, we found in colloid extracted from single follicles a proteolytic activity which increased with activation of the gland and decreased under opposite conditions. These observations led to the postulation of an enzymatic theory for the reabsorption of the follicular colloid.

Similar results were found in normal and pathological human thyroids. In severe toxic goiters, there is an increase of P.A. of about 100 per cent above normal. In simple colloid goiter, it is diminished to about —30 per cent. These results seem to indicate that the proteolytic system may have an important role in the physiopathology of these diseases.

Results on mild toxic goiters treated with iodine, together with previous experimental results, lead to the conclusion that probably the therapeutic action of iodine may be attributed to its influence upon the proteolytic system of the thyroid gland.

Experiments of iodination *in vitro* show that this action is possibly due to a direct iodination of the proteolytic enzyme.

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### *Discussion of the Paper*

DR. C. P. LEBLOND (*McGill University, Montreal, Canada*):

The statement that the vacuoles and staining reactions of the colloid are artifacts should be qualified. It is true that they actually are artifacts since, after osmic-acid or freezing-drying fixation, the colloid shows no vacuoles and does not stain differentially with trichrome stains. However, in sections fixed with the usual fixatives, both the vacuoles and the staining reactions of the colloid have a definite meaning, since they vary considerably according to the physiological condition of the gland. The vacuoles, for instance, which are absent in the living animal and occur during the process of fixation, are most numerous in stimulated thyroids, but rare in resting glands. Similarly, the colloid is predominantly basophilic in activated glands and acidophilic in resting glands. Therefore, it may be concluded that the presence of vacuoles and basophilia in the colloid corresponds to properties of the colloid usually found in activated glands.

Furthermore, the behavior of the basophilic and acidophilic colloid toward radio-iodine is quite different. For instance, 24 hours after administration of a small dose of radio-iodine, the acidophilic follicles contain much more radio-iodine than the basophilic ones, contrary to what is found earlier after injection. This finding was interpreted as indicating that the turnover of iodine is more rapid in the basophilic follicles than in the acidophilic ones.

If colloid is smeared from a sliced thyroid (dog) onto a glass slide, this colloid may be fixed and stained and will appear either basophilic or acidophilic or show a variegated appearance, according to the state of the gland. If, in these conditions, acidophilic colloid is treated with trypsin, it will rapidly be transformed into basophilic colloid. From this experiment, it may be suggested that the basophilia is somehow connected with the presence of proteolytic enzymes, since Dr. De Robertis showed that the activated (predominantly basophilic) thyroids do contain an increased amount of proteolytic enzyme.

DR. VICTOR M. TRIKOJUS (*University of Melbourne, Melbourne, Australia*):

Reference was made to the work of Wright and Trikojus,<sup>1</sup> who demonstrated that iodine inactivation of the thyrotrophic hormone, presumably by oxidation, resulted *in vitro* with molecular iodine under physiological conditions as regards pH and temperature. (Similar findings have been recorded by Rawson and colleagues.) As the thyrotrophic hormone is known to exert its effect intra-thyroidally, the possibility of the favorable influence of iodine in Graves' disease being associated with inactivation of the thyrotrophic hormone should not be overlooked.

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DR. E. D. GOLDSMITH (*New York University, New York, N. Y.*):

With reference to Dr. De Robertis's observation that sulfanilamide does not inactivate peroxidase, it is of interest to recall that we had reported (Growth **9**, 1945) that sulfadiazine and para-aminobenzoic acid exerted no action on the effects of thyrotrophic hormone in *Rana pipiens* larvae. Two mg. of sulfadiazine and 1 mg. of para-aminobenzoic acid in 0.05 cc. distilled water were injected pleuro-peritoneally on alternate days. Similar treatment with 1 mg. of thiourea did antagonize the effects of thyrotrophic hormone in amphibian development. Similarly, in the chick, sulfonamides have been found ineffective as an antithyroid agent. However, Dr. MacKenzie can tell us more about that.

DR. C. G. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*):

I wonder if an alternative explanation of the effects of thiourea and sulfanilamide on thyroid peroxidase is not permissible. Recently, Randall has shown that thioureas, far from inhibiting horse-radish peroxidase, are themselves substrates for the peroxide-peroxidase system. If the postulated thyroid peroxidase is similar to this one, it seems that thioureas, rather than inhibiting it, may compete with iodide for the enzyme-peroxide complex, and also reduce any oxidized iodine as fast as it is formed.

With respect to the sulfonamides, Lipmann reported that they were not oxidized by horse-radish peroxidase plus peroxide, but that they inhibited the oxidation of PABA, and that  $H_2O_2$  did not disappear in their presence. This suggests that they inhibit the enzyme in a non-competitive fashion.

DR. E. DE ROBERTIS:

Although other interpretations of the mechanism of inhibitors can be supported, the one favored by us seems to explain better our experimental results.

The inhibiting effect of thiourea on vegetable-peroxidase is a well

known fact as stated by Sumner and Somers.<sup>1</sup> In our experiments the histochemical reaction of peroxidase with benzidine was inhibited with 0.001 M thiourea, while the unspecific reaction shown by hemoglobin was "inhibited" only with 0.03 M. Furthermore, in our experiments on the liberation of iodine from iodides, 0.03 M thiourea was needed to prevent the formation of free iodine in a peroxide-iodide system, while 0.001 M thiourea stopped the reaction when peroxidase was also present. These facts can be explained by the extreme rapidity with which peroxide and peroxidase unite together to form a relatively tight complex.<sup>2</sup>

Mann and Keilin<sup>3</sup> have shown that sulfonamide does not inhibit peroxidase even at 0.01 M concentration, and Wood<sup>4</sup> demonstrated that sulfonamide may act in a competitive way on the oxidation of *p*-aminobenzoic acid with peroxidase. It is also known that, when catechol is oxidized by tyrosinase, an *o*-quinone is formed which may react with sulfanilamide to form a red compound. The facts observed by us—that sulfa-drugs do not lower the potential redox (as does thiourea), that they do not inhibit the histochemical reaction with benzidine, that sulfathiazole with iodine forms a yellow compound, and that sulfanilamide injected into the animal produces a reddish tinge in the thyroid gland—can be better explained by the supposition that these compounds act in a competitive way with the "essential metabolite."

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# THE CHEMICAL CYTOLOGY OF THE THYROID GLAND

By EDWARD W. DEMPSEY\*

*Department of Anatomy,  
Harvard Medical School, Boston, Massachusetts*

**I**N recent years, rapid strides have been made in applying procedures for chemically characterizing cells and cellular inclusions. A potentially great advantage resides in these procedures in that they reveal the location of metabolically active substances in tissues and cells. Experience has shown that many such substances are sharply segregated, so that their local or effective concentrations may be many times greater than their apparent concentrations as judged by analyses of tissue homogenates.

Although there are many inherent advantages in a combined chemical and cytological approach, not enough information is presently available on which to base far-reaching generalizations. Practical difficulties, the non-specific or semi-specific character of many of the methods, and the relative newness of the field all contribute to a lack of sufficient trustworthy data. Consequently, at the present moment, one can only tentatively make correlations and draw conclusions which may be useful in constructing working hypotheses, but which cannot be regarded as sufficiently well established for a more solid theoretical structure.

*General Histophysiological Considerations.* The parenchymatous portion of the thyroid is derived from a median downgrowth of entodermal cells from the tongue which proliferate to form cords or sheets. These cells become separated into small masses, in the center of which a lumen appears. Thus, the mature thyroid gland consists of a single layer of epithelial cells arranged in follicles which contain a transparent gelatinous colloid. The follicular cells may be columnar, cuboidal or flat, depending upon whether small, moderate or large amounts of colloid are present. Occasional cords or groups of cells without lumens occur between the follicles and constitute the interfollicular cells.

The follicular cells exhibit well-defined mitochondria, Golgi nets, and intracellular vacuoles. Both mitochondrial and Golgi material increase in states of hyperactivity. The Golgi apparatus normally lies above the centrally-located, rounded nucleus. It has been claimed that this apparatus reverses its position and becomes infranuclear when the follicle is activated, but modern work has not confirmed this notion. Lipoid-containing vacuoles are present in the apices of the follicular cells. During active states, the amount of intracellular lipoid increases. Other vacuoles, containing a colorless fluid,<sup>44</sup> the tinctorial properties of which are

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similar to those of the intrafollicular colloid,<sup>3</sup> also occur within the cells. Gersh and Caspersson<sup>15</sup> have observed more recently that ultra violet light is absorbed comparably by these vacuoles and by the colloid.

The follicular cells rest upon a delicate network of argyrophilic reticular tissue (FIGURES 6 and 7). There is no well-defined basement membrane, although a thin homogenous line is stained with the azan procedure. The follicular cells may easily become detached and float free into the colloid. Occasional, usually solitary, mast cells are scattered in the reticular framework of the gland in some animals. A rich vascular plexus permeates the stroma of the gland, surrounding the individual follicles by a network of anastomosing capillaries. These capillaries frequently indent the basal cytoplasm of the follicular cells so that their courses appear to be intraepithelial.

The basic pattern of the thyroid gland is similar in all vertebrate animals. The relative proportions of cells and colloid appear to constitute the principal variations among the various species. Thus, the rhesus monkey has large follicles plentifully filled with thick, intensely chromophilic colloid and an excessively thin and flat epithelial layer, whereas in the mouse and rat the follicular cells are relatively tall and the colloid is correspondingly meager and thin in consistency. A well-developed zonation occurs within the thyroid of these last two species. The peripherally located follicles are among the largest in the gland and contain dense colloid which stains blue with the eosin methylene blue method and red with the triacid azan. The central portion of the thyroid, on the contrary, has small follicles, the colloid of which is scanty and stains weakly with basic dyes, and with the blue, rather than the red, component of the azan stain. The cells of these central follicles are high cuboidal or columnar in shape. Local variations in appearance also characterize the human thyroid. Microfollicular and macrofollicular areas are present, and occasionally regions or cushions of tall columnar cells occur within follicles with otherwise flattened epithelium.

Alterations in the microscopical appearance of the thyroid gland may be induced in animals by appropriate experimental procedures. Hypophysectomy in the rat is followed by shrinkage in the total size of the gland. Individual follicles, however, contain increased amounts of colloid and the follicular cells become excessively flattened.<sup>39</sup> On the other hand, administration of pituitary extracts containing the thyroid-stimulating principle, thyrotropin, depletes the intrafollicular colloid and increases the height of the follicular cells. Similarly, Cramer<sup>6</sup> has shown that exposure to a hot environment causes an increased deposition of colloid, whereas cold induces discharge of the colloid into the circulation. Corresponding and concomitant alterations in the Golgi apparatus, mitochondria, and cell-height attend these experimental procedures. These effects of environmental temperature apparently are mediated through the anterior pituitary gland, since the expected changes do not occur in hypophysectomized rats or in rats in which the pituitary stalk

has been transected.<sup>43</sup> These findings have been interpreted to mean that the thyroid actively secretes its stored colloid under the stimulus of pituitary thyrotropin and that colloid is stored when the thyrotropin is decreased. Such an interpretation is in good accord with pathological observations which have correlated hyperthyroid states with depleted colloid stores and hyperplastic epithelium.

Recently, drugs which profoundly modify the activity and appearance of the thyroid have been discovered.<sup>1-3, 34, 37</sup> These drugs, for example thiouracil, prevent the synthesis of hormone by the thyroid gland but do not affect the peripheral action of thyroid hormone, nor do they interfere with the pituitary-induced hyperplasia of the gland. These inferences are drawn from Astwood's<sup>3</sup> and the MacKenzies'<sup>34</sup> experiments, which establish the facts that thiouracil causes a profound hyperplasia and loss of colloid in normal but not in hypophysectomized rats, and that the hyperplastic effects of the drug may be nullified by concomitant administration of thyroid hormone. Indeed, Dempsey and Astwood<sup>8</sup> found the latter effect to be so striking that it provides a method sensitive enough to detect altered rates of thyroid secretion in rats maintained in hot and cold environments. Mixner, Reineke, and Turner<sup>36</sup> have obtained similar results in different breeds of fowl.

To summarize these various histophysiological data, it may be said that morphological changes occur which characterize states of increased or decreased thyroïdal activity. Differences in the morphological appearances of the thyroids of various animals suggest that some, such as the monkey, have low rates of activity, whereas others, for example the rat, have high rates. Experimental procedures, involving subsection of rats to hot and cold environments, hypophysectomy and replacement therapy with thyrotropin, and treatment with antithyroid drugs and with thyroid hormone, permit the regulation of thyroid activity at any desired level. The thyroid gland, therefore, is an admirable organ for chemocytological analysis, since its histophysiological variations are well understood and its physiological activity may be regulated easily.

*Basophilic Substances of the Thyroid Gland.* In eosin-methylene blue preparations of the thyroid gland, various objects exhibit basophilic properties. The follicular cells contain basophilic nuclei, and, in addition, a fine stippling of basophilic substance occurs in the cytoplasm. The thick, inspissated colloid of large follicles stains deeply blue, whereas the thinner colloid of more active follicles becomes faintly or moderately colored (FIGURES 1 and 3). Finally, the moderately abundant mast cells of the thyroid stroma contain granules which stain intensely with methylene blue.

The nuclei of the follicular and stromal cells stain positively with the Feulgen procedure. The Feulgen reaction is usually regarded as specific for the nuclear nucleoproteins which contain desoxyribose.<sup>40</sup> The nuclei of peripheral, inactive follicles in the rat thyroid stain slightly more

deeply than do those of the central, active follicles.<sup>9</sup> Similarly, stimulation of the thyroid by exposure of rats to cold and by the administration of thiouracil causes a slight diminution in the Feulgen reaction of the nuclei of the follicles. Presumably, therefore, states of active secretion lead to a slight decrease in the nuclear Feulgen reaction.

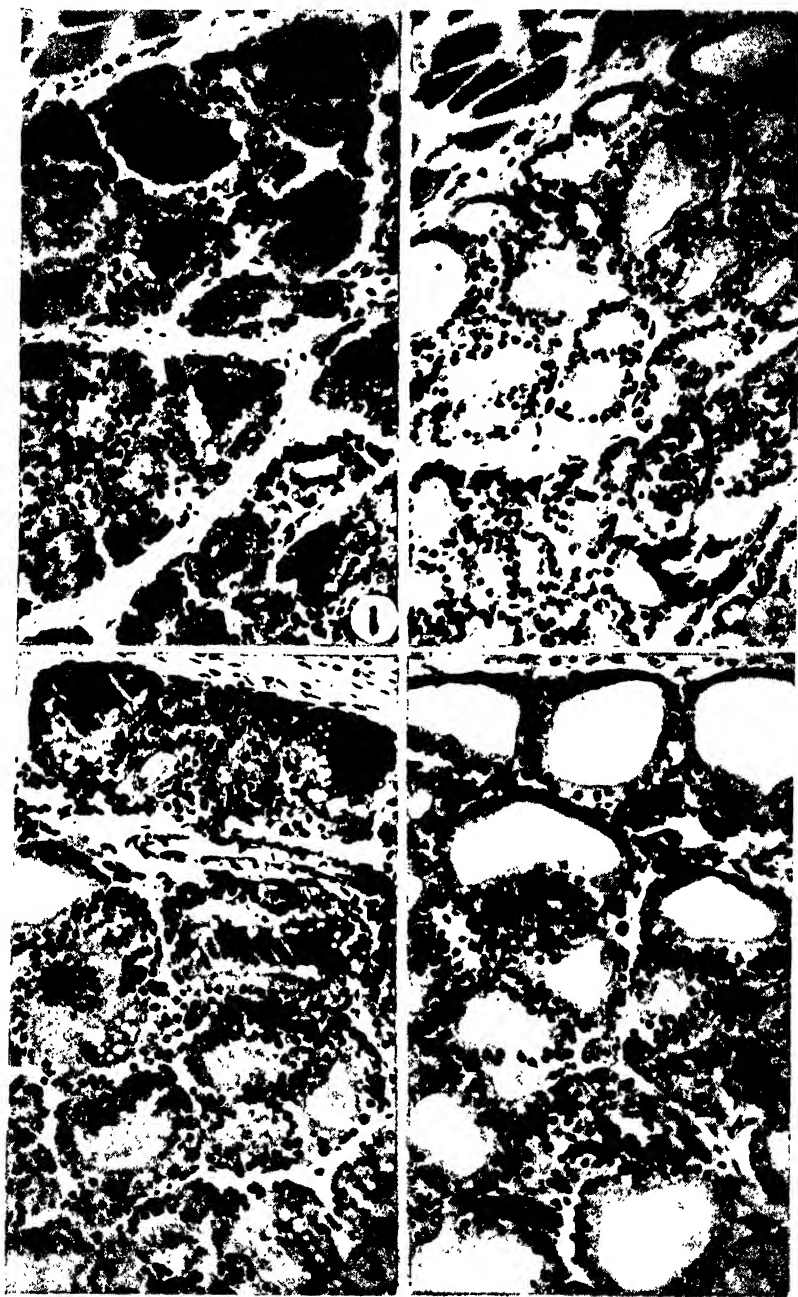
The cytoplasm of the follicular cells, the intrafollicular colloid, and the mast cell granules are all negative when tested by the Feulgen procedure. The intrafollicular colloid frequently takes on a purplish tint in Feulgen preparations. This does not represent a positive reaction, however, since a similar tinge occurs when thyroids are stained by the Bauer<sup>1</sup> procedure for glycogen and by the plasmal reaction of Feulgen and Voit.<sup>13</sup> The essential difference in these various reactions concerns the kind of hydrolysis to which the tissue is subjected prior to treatment with Schiff's leucofuchsin reagent. The specificity of the Feulgen reaction derives from the character of the initial hydrolysis in warm hydrochloric acid; consequently, the purplish tinge referred to above cannot be ascribed to the presence of desoxyribonucleoprotein in the colloid. The results obtained with the Feulgen reaction, therefore, indicate that the nuclear basophilia of the thyroid cells can be satisfactorily accounted for by their content of desoxyribonucleoprotein, but that the basophilic properties of the cytoplasm, colloid, and mast-cell granules must find some other explanation.

Cytoplasmic, or ribose-containing, nucleoproteins may be characterized in other locations by their lability to digestion by ribonuclease.<sup>10</sup> After incubation of sections of thyroid gland in solutions of crystalline enzyme, the follicular cytoplasm and the colloid both fail to stain with methylene blue (FIGURES 2 and 4). On the other hand, the chromatin of the nuclei and the granules of the mast cells are unaffected by treatment with the enzyme.

The destruction of basophilia in the cytoplasm and colloid by digestion in ribonuclease strongly suggests that these regions contain appreciable concentrations of ribonucleoproteins. However, such a conclusion is open to the criticism that ribonuclease may be non-specific in its action and may, in addition to depolymerizing ribonucleoproteins, also cause changes in other proteins of fixed tissues. Fortunately, this possibility may be tested by a second method for characterizing nucleoproteins.

The staining reactions of proteins under controlled physicochemical conditions may serve to characterize certain substances. Solutions of  $5 \times 10^{-4}$  molar methylene blue were adjusted to various pH values with acetate or phosphate buffers having an ionic strength of 0.01. Sections of Zenker-fixed thyroids were placed in 1500 cc. of these solutions and stained at  $25 \pm 0.1^\circ$  C. for 24 hours, during which time equilibrium was attained. The sections were rapidly dehydrated and covered as permanent preparations. The intensity of staining of the various tissue elements was determined by projecting the image of the section upon the





FIGURES 1-4 (See opposite page)

ground-glass viewing plate of a photomicrographic camera. The amount of light transmitted through a given portion of the tissue was measured with a Model 512 Photovolt light meter, the search unit of which was fitted with an extension piece having an aperture  $\frac{1}{8}$  inch in diameter. The light absorbed was calculated as per cent and plotted against pH to form a graph relating staining affinity to the acidity of the staining solution (FIGURE 5).

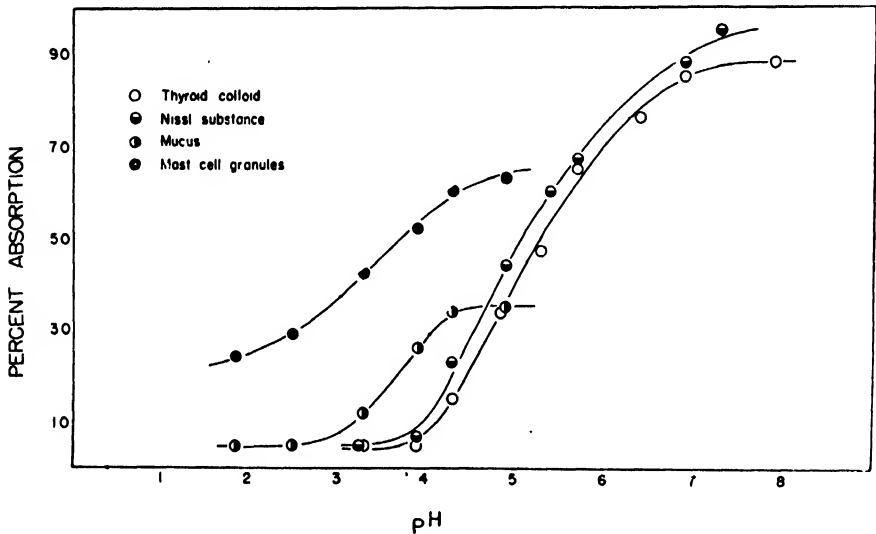


FIGURE 5. A comparison of the binding capacity for methylene blue of several basophilic substances over a range of pH. Nissl substance, a known ribonucleoprotein, and thyroid colloid fail to stain below pH 4.0 and attain a maximal uptake of dye at about pH 7.0. On the other hand, mucus and the granules of mast cells continue to stain in solutions considerably more acid than pH 4.0. This behavior presumably indicates more strongly dissociating acid groups in the latter two substances.

Curves constructed for thyroid colloid indicate that staining affinity increases sharply between pH 4.0 and pH 7.0. Below pH 4.0, therefore, the negative charge of the colloid is suppressed by the acidity of the staining solution, whereas at and above pH 7.0 the negative charge has attained its maximum. For comparison, a similar curve constructed for

#### FIGURES 1-4 (See opposite page)

The effect of ribonuclease upon the basophilia of the thyroid gland. The four sections on this plate were prepared from Zenker-fixed thyroids and were stained identically by the eosin-methylene blue procedure. The photographs were exposed, developed, and printed for identical times. All sections X 200.

FIGURE 1. Thyroid gland from normal control rat. The peripheral follicles (upper part of figure) contain intensely basophilic colloid.

FIGURE 2. Section adjacent to that presented in FIGURE 1, and treated identically except for digestion in a solution of crystalline ribonuclease. The basophilic reaction of both colloid and cytoplasm is greatly depressed.

FIGURE 3. Thyroid gland from a rat maintained in a cold room. The basophilia of the colloid is reduced as compared with that of the control rat shown in FIGURE 1.

FIGURE 4. Section adjacent to that presented in FIGURE 3, and treated identically after digestion in ribonuclease. The basophilia of the colloid has been abolished.

Nissl bodies from nerve cells in which the presence of nucleoprotein has been well substantiated indicates a similar suppression of staining below pH 4.0 and a maximum uptake of basic dye at about pH 7.0. Consequently, the position and character of the curve relating pH to staining capacity provide evidence that nucleoprotein is a constituent of thyroid colloid. This conclusion fortifies the similar one derived from digestion of sections with ribonuclease. It appears, therefore, that the basophilic properties of the colloid of the follicular cytoplasm may be accounted for by their content of ribonucleoproteins.

The remaining basophilic element of the thyroid, namely, the mast cell, is colored metachromatically with methylene blue and also by toluidin blue after Holmgren and Wilander's<sup>29</sup> procedure involving fixation in basic lead acetate. According to these authors, such metachromatic staining indicates the presence of sulfate-containing mucopolysaccharide complexes. The nuclei and cytoplasm of the follicular cells and the intrafollicular colloid, on the other hand, do not stain metachromatically. The metachromatic basophilia of mast-cell granules is unaffected by digestion in ribonuclease. The signature of these granules, when obtained by measuring their affinity for methylene blue at various pH values, is strikingly different from those of colloid and nuclei (FIGURE 5). The granules continue to stain appreciably in solutions of pH 1.8, indicating much stronger dissociation of their negative charges than is the case for those of nucleoproteins, a fact consonant with the thought that half-esters of sulfuric acid are an integral part of their composition. These observations indicate that the granules of mast cells do not exhibit the expected reactions of nucleoproteins but behave in a fashion characteristic of sulfate-containing mucopolysaccharide substances.

In summary of the above, the basophilic properties of the various thyroid components may be satisfactorily correlated with their other chemical characteristics. The nuclei apparently contain desoxyribonucleoproteins. The cytoplasm of the follicular cells and the intrafollicular colloid react like ribonucleoproteins. The granules of mast cells exhibit behavior characteristic of mucopolysaccharides.

*Autofluorescence of the Thyroid.* The fluorescence of tissues, both in their natural state and after reaction with fluorochromes, has been exploited only very slightly by histologists. The thyroid gland is no exception to this statement, there being few studies available on thyroidal fluorescence. Hartoch<sup>27, 28</sup> has studied the fluorescence of the thyroid of the living rat with fluorochromes. Hamperl<sup>29</sup> has investigated the autofluorescence of human thyroids, and Grafflin has published a series of observations on the deer,<sup>18, 19, 22</sup> the gorilla,<sup>20</sup> the baboon,<sup>21, 24</sup> the chimpanzee,<sup>24</sup> and the elephant.<sup>23</sup> Dempsey<sup>7</sup> investigated the autofluorescence of the rat thyroid after various fixatives and physiological procedures.

The connective tissue framework of the thyroid is fluorescent in all of the species studied. There is good agreement among the various authors

as to its bluish or bluish-white color. In general, the denser capsule fluoresces more intensely than do the more delicate strands of interfollicular tissue. Occasional pigment bodies are scattered among the stromal cells in many species. These pigmented areas exhibit a reddish or brownish-yellow fluorescence, and have been designated as "wear and tear" pigment (*Abnutzungspigment*) by Hamperl<sup>26</sup> and Grafflin.<sup>18</sup>

The fluorescence of colloid varies greatly in individual follicles and in diverse animals. Moreover, according to Dempsey,<sup>7</sup> the previous chemical treatment involved in the kind of fixation also determines the kind of fluorescence observed. After formaldehyde, which Hamperl<sup>26</sup> and Grafflin<sup>18</sup> have adopted as the fixative of choice, the majority of the follicles contain colloid which fluoresces weakly or not at all. Occasional follicles, increasing in number with age and correlated with increased density of the colloid, fluoresce more intensely with a bluish or bluish-white color, sometimes with a greenish component. This fluorescent colloid may be dispersed evenly in the follicle or may occur as irregular granules and masses within a matrix of weakly fluorescent colloid. The distribution of this highly fluorescent colloid is similar, in several species, to the distribution of dense, basophilic colloid which also exists variably as granules, as masses, or homogeneously throughout individual follicles. There seems good reason, therefore, to equate the strong fluorescence of these areas with the more dense aggregation of the colloid in some follicles.<sup>18</sup>

In addition to the fluorescence of the colloid itself, Grafflin<sup>22</sup> has described crystals which fluoresce a yellowish color and which occur occasionally in the follicles of the deer thyroid. The nature of these crystals is undetermined.

The fundamental fluorescent color of the follicular cells appears to be an ill-defined bluish or bluish-white. The intensity varies considerably in different species. In the baboon the cells are practically non-fluorescent,<sup>21</sup> whereas in the rat the intensity is high and of a whitish quality.<sup>7</sup> Most animals show, in addition, a yellowish fluorescence of a granular character, concentrated for the most part in the apical portions of the follicular cells. The distribution of these yellowish granules corresponds to that of yellow pigment granules, which may frequently be observed in the thyroid epithelium. It would appear that young animals contain smaller numbers of these yellow granules than do older animals. This and other considerations have led Hamperl and Grafflin to the conclusion that they represent intracellular "wear and tear" pigment.

A brownish pigment which is preserved in formaldehyde but is destroyed by weak solutions of KOH has been observed by Grafflin<sup>20</sup> in both the parenchymatous and the supporting tissues of the thyroid of the gorilla and baboon. This pigment fluoresces a well-defined red color, a circumstance which suggests its possible porphyrin nature.

Although the fluorescence of the colloid in the majority of the follicles is slight after fixation of the thyroid in formaldehyde, fluorescence is

greatly enhanced after fixation in alcohol.<sup>7</sup> Moreover, the intensity of fluorescence varies considerably in different physiological states. The stored colloid of inactive follicles fluoresces intensely, whereas weaker reactions occur in follicles from animals maintained in cold environments. Weak fluorescence also characterizes the small residuum of colloid in the thyroids of animals to which thiouracil had been administered. The reconstituted colloid which accumulates after hypophysectomy is also deficient in fluorescence, suggesting that the fluorescence may be related to the hormone concentration in the gland, since the colloid produced after these experimental procedures is deficient in iodine.

*Iron Reactions of the Thyroid.* Iron may be demonstrated in tissue sections by two principal procedures, namely, by Turnbull blue or Prussian blue reactions, in which iron enters into reactions with a colored end-product, and by the observation of a yellow, orange or red ash in microincinerated specimens.<sup>38</sup> The color reactions are precise and satisfactory, but unfortunately only part of the total iron contained in the tissue is revealed by them. Microincineration causes drastic bubbling of the tissue with, consequently, the possibility of rearrangement of the various components. The cytological location of iron as revealed by this method is therefore open to question. Despite this fault, the total quantity of iron, metallic or organic and masked or unmasked, is unquestionably placed in evidence.

Iron stainable by the Prussian blue or Turnbull blue procedures is usually absent in the thyroid. However, occasionally, a single follicle or an area composed of a few follicles with their adjacent stroma will be observed to stain. In these rare areas, the stain may be barely perceptible, or, upon occasion, be fairly intense. Macrophages containing hemosiderin are usually present in the stroma contiguous to these areas. There seems little reason to doubt that such stainable iron in the thyroid represents the remnants of extravasated blood from accidental hemorrhages.

A somewhat different picture is revealed in microincinerated specimens. Grafflin<sup>18</sup> has provided a careful account of the location of red or yellow ash in incinerated specimens of the thyroid from a Barasingha deer. The colloid is typically poor in ash and almost invariably devoid of iron, except for occasional rare follicles which exhibit a few granules. In the follicular cells, granular iron is present in the cytoplasm of many of the follicular cells. These iron granules are apically located for the most part, and are variably concentrated in different regions of the gland. Both their location within the cell and their variable distribution suggest the similar positions of the yellow fluorescent pigment of the deer thyroid. The nuclei are poor in ash and contain no recognizable iron.

A similar situation prevails in the rat thyroid. The colloid is typically poor in ash, especially in the central, active follicles. The larger periph-

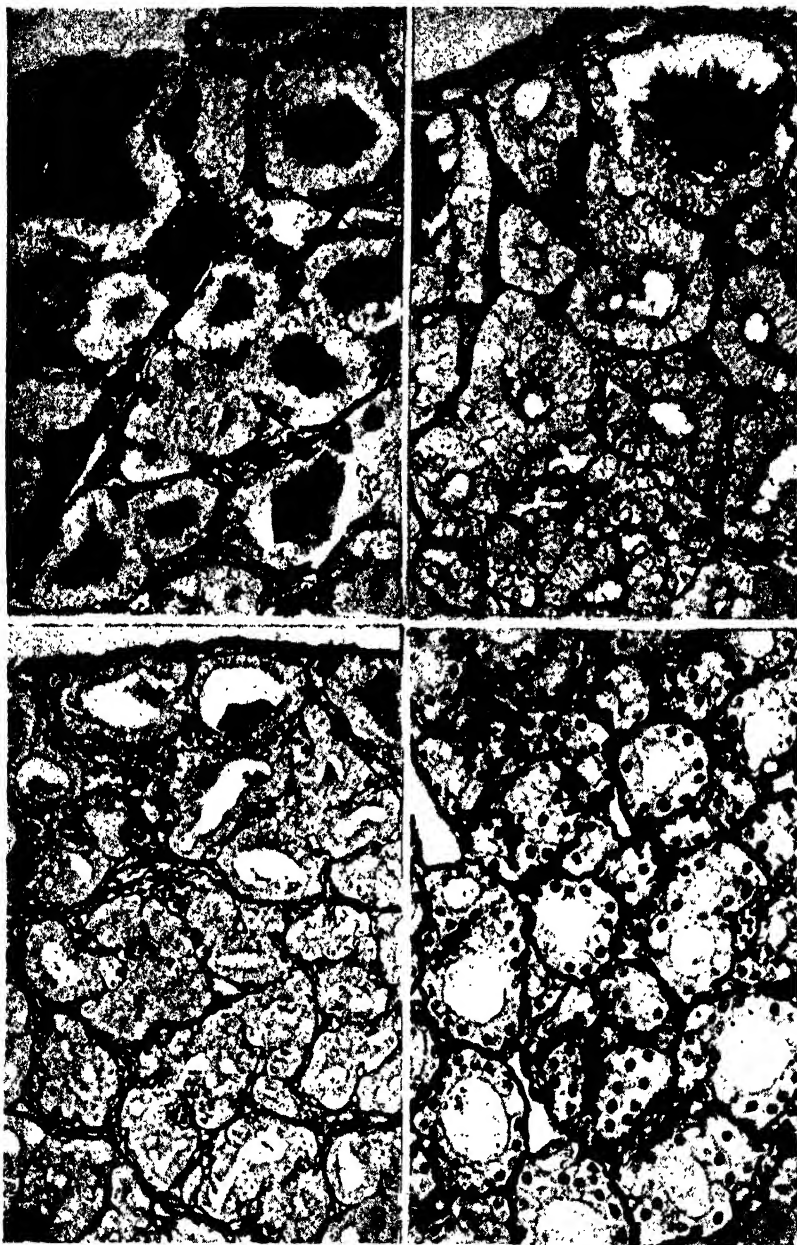
eral follicles, the colloid of which is denser and more basophilic, exhibit considerably greater quantities of ash, predominantly blue in color, although a few yellow or orange granules usually occur in each follicle. The follicular cells, on the other hand, contain considerable quantities of yellow ash which typically occur in the apical margins of the cells. The rat thyroid does not contain yellow fluorescent granules. Consequently, it would seem that the iron content of the follicular cells must represent some constantly occurring substance rather than reflect the presence of some adventitiously occurring pigment.

*Lipoids of the Thyroid.* Sudanophile material has been recognized as a regular component of the thyroid gland for many years. Langendorf<sup>31</sup> first described it in the follicular epithelium of the calf and dog. Fat is commonly believed to increase in amount with advancing age, although there is no complete agreement upon this point. According to Grafflin,<sup>18</sup> the follicular cytoplasm of the deer thyroid contains numerous small, intensely stained sudanophile droplets. The predominant position of these granules is in the apical portions of the cells. They may, however, extend down into the paranuclear region and may even be located basal to the nucleus. The picture in different follicles is surprisingly uniform, the principal variations being in the size of the lipoid droplets. From the appearance of sections stained with sudan III, one would be forced to conclude that there is only one type of cell in the follicular epithelium.

The rat thyroid gland also contains sudanophile droplets in the follicular epithelium. The droplets are predominantly located above the nucleus in a distribution similar to that described by Grafflin for the deer. Stimulation of the thyroid by subjecting rats to a cold environment or by administering thiouracil results in a prompt increase in the size and number of the sudanophile material.

*Argyrophilia and Schiff Reactions of the Thyroid.* In an attempt to determine the possible presence of glycogen in the thyroid, Dempsey and Singer<sup>9</sup> examined the glands of rats and cats which had been prepared by Bauer's<sup>4</sup> and Mitchell and Wislocki's<sup>35</sup> methods. Bauer's method involves staining glycogen, after appropriate fixation, by its reaction with Schiff's reagent. Mitchell and Wislocki showed that glycogen preserved in sections is stained an intense black by ammoniacal silver nitrate. The intrafollicular colloid stains with both procedures, although in the case of the silver reaction the colloid is colored brown rather than the jet black characteristic of glycogen.

Despite the occurrence of these reactions in the thyroid gland, the reactive substance is not glycogen. Digestion of the sections with saliva does not abolish either the Bauer or the silver reaction. Since both reactions ordinarily indicate active carbonyl groups, there is a strong likelihood that the intrafollicular colloid contains some substance, preserved by protein precipitants, characterized by reducing activity equivalent to that of aldehydes.



The sections on this plate were prepared by Pap's ammoniacal silver nitrate method. They demonstrate the nature of the reticular framework of the gland, and also illustrate the argyrophilia of the colloid.  
(For description see facing page)

The reducing activity of the colloid varies in different stages of thyroïdal activity. In the glands from normal rats, the strongest reactions invariably occur in the large peripheral follicles containing the densest colloid (FIGURE 6). In the centrally located follicles, where the epithelium is higher and the colloid is less dense, the reactions are weak. Correspondingly, in animals exposed to cold (FIGURE 7) or treated with thiouracil (FIGURE 8), the reactions decline in intensity or fail completely, depending upon the degree of thyroïdal activation.

In addition to the reaction of the colloid with ammoniacal silver nitrate, argyrophilic inclusions can be demonstrated in the follicular epithelium by two other silver methods. Argyrophilic granules, varying in size from dust-like in the rat to an appreciable size in the dog, are displayed in sections prepared by Bodian's protargol method (FIGURE 13). These granules are distributed rather evenly throughout the cytoplasm, being absent only in small vacuoles in the apical portions of the epithelium. With this method, the colloid is entirely unstained. Granules, similarly located in the follicular epithelium, are also revealed in sections fixed in Zenker's acetic fluid and stained by Pap's ammoniacal silver procedure. These granules exhibit no very clear alteration in number or appearance in the activated glands from rats exposed to cold temperatures (FIGURES 10 and 11).

One further comment can be made concerning the chemical behavior of the substance responsible for the carbonyl reactions of the colloid. Sections immersed directly in Schiff's reagent, which does not call for previous hydrolysis, exhibit a moderately intense violet stain in the colloid (FIGURE 12). It would appear, therefore, that the carbonyl groups are exposed and do not require preliminary unmasking by hydrolysis. Furthermore, strong acid hydrolysis, for example with tenth normal hydrochloric acid at 60°C. as in Feulgen's procedure for desoxyribonucleoproteins, lessens the intensity of the reaction or prevents it completely. The faint coloration observable in the colloid after short hydrolysis in Feulgen's procedure led Uotila<sup>42</sup> to suggest that desoxyribose nucleoproteins occurred within the follicle. It seems clear, from the above account of the reactions involving modifications of the Schiff reaction, that the staining of the colloid observed by Uotila did not represent a true Feulgen reaction for nucleoproteins, but was caused rather by the presence of the free carbonyl groups, which account for the Schiff reaction.

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FIGURES 6-9 (See opposite page)

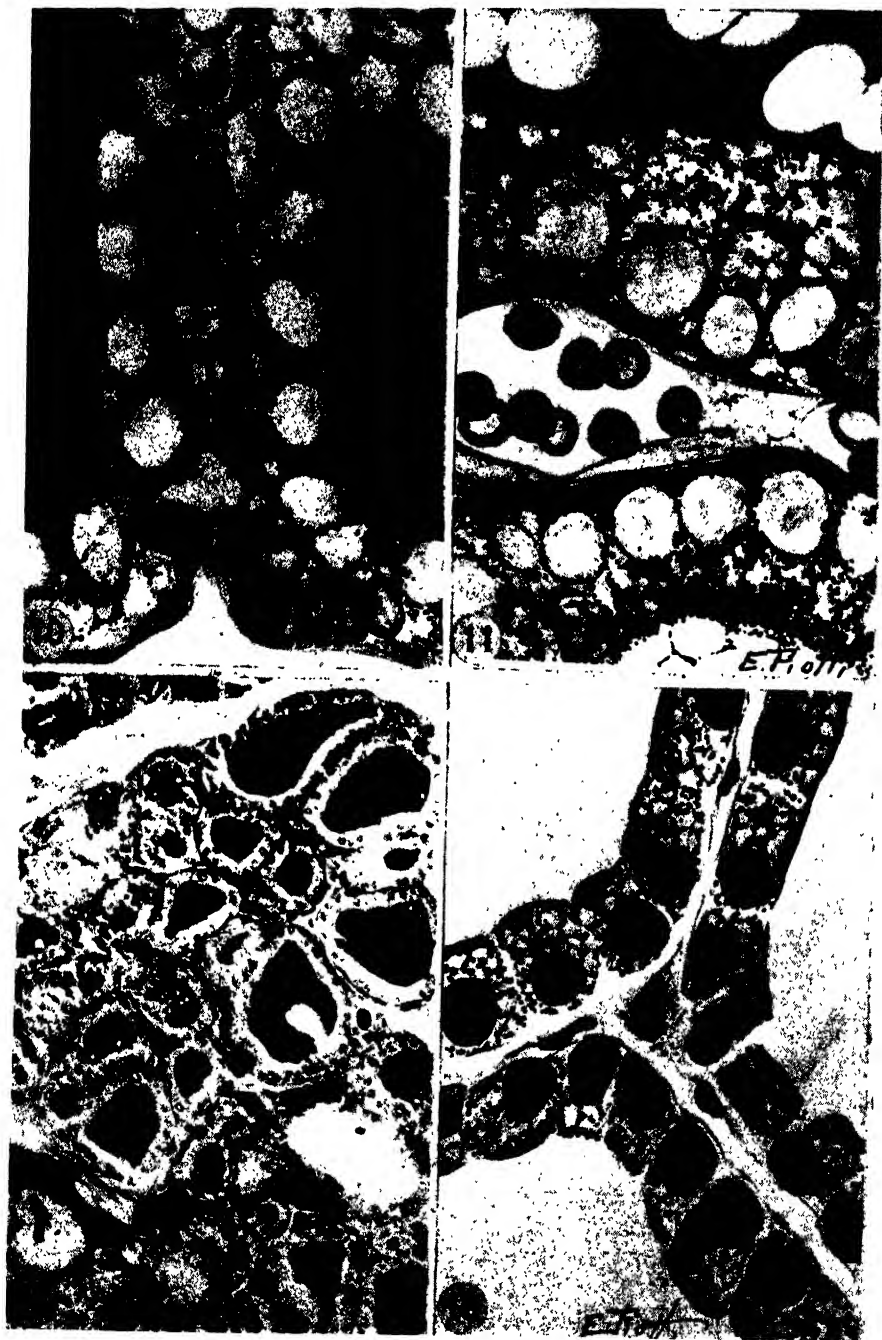
FIGURE 6. Thyroid gland from normal control rat. Bouin fixation, section digested in saliva before staining. X 350.

FIGURE 7. Section illustrating the reduction in argyrophilia of the colloid in a rat exposed to cold. Treatment of the section same as that in FIGURE 6. X 350.

FIGURE 8. Section illustrating the further reduction in argyrophilia after administration of thiouracil. Treatment of section same as that in FIGURE 6. X 200.

FIGURE 9. Abolition of argyrophilia of the colloid after fixation in formaldehyde. Cat thyroid gland. X 350.





FIGURES 10-13 (For description see facing page)

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*Enzyme Reactions of the Thyroid Gland.* A number of enzyme systems have been located in the thyroid by chemocytological procedures. The more prominent of these involve oxidase, peroxidase, phosphatase, and proteolytic activities.

Treatment of frozen sections of rat thyroid with the nadi reagents (paraphenylenediamine and alphanaphthol) leads to the rapid appearance of blue granules scattered throughout the parenchymal cells. This reaction, frequently called the indophenol oxidase reaction, has been studied by Keilin,<sup>30</sup> who regards it as caused by the activity of the cytochrome oxidase-cytochrome *c* system. Dempsey<sup>7</sup> found that the indophenol oxidase activity of rat thyroid was not abolished by the presence of the antithyroid drug, 2-thiouracil. On the other hand, using manometric methods, Franklin, Lerner, and Chaikoff<sup>14</sup> have reported a depression in the respiration of thyroid slices after poisoning with thiouracil and other antithyroid substances.

Peroxidase activity is also demonstrable in the rat thyroid. Blue granules appear in the follicular cells after immersion of frozen sections in mixtures of benzidine and hydrogen peroxide.<sup>7</sup> Unlike the nadi reaction, the peroxidase activity is easily suppressed by concentrations of thiouracil as low as one part in ten thousand. These observations have been challenged by Glock,<sup>17</sup> who found much of the peroxidase activity of minced thyroid tissue in a fraction containing hemoglobin. Although he was unable to identify a peroxidase characteristic for the thyroid epithelium, Glock's experiments do not disprove the possibility that such an enzyme exists. De Robertis and Grasso,<sup>12</sup> also using chemocytological methods, have confirmed Dempsey's observations, both as to the intracellular location of peroxidase and its inhibition by antithyroid drugs. These authors have also studied the oxidation reduction potential of the thyroid follicle and find it greatly reduced after administration of thiouracil.

De Robertis,<sup>11</sup> in an interesting series of experiments, has shown that a proteolytic enzyme may be demonstrated in the colloid from active thyroid follicles. The amount of this enzyme increases after the administration of thyrotropin, and causes liquefaction of the dense thyroid colloid. Such liquefaction presumably is useful in releasing the stored colloid during active secretion.

The location of phosphatases in the rat thyroid gland has been studied by Dempsey and Singer.<sup>9</sup> Although the concentration of neither enzyme is very high, both acid and alkaline glycerophosphatases occur in the

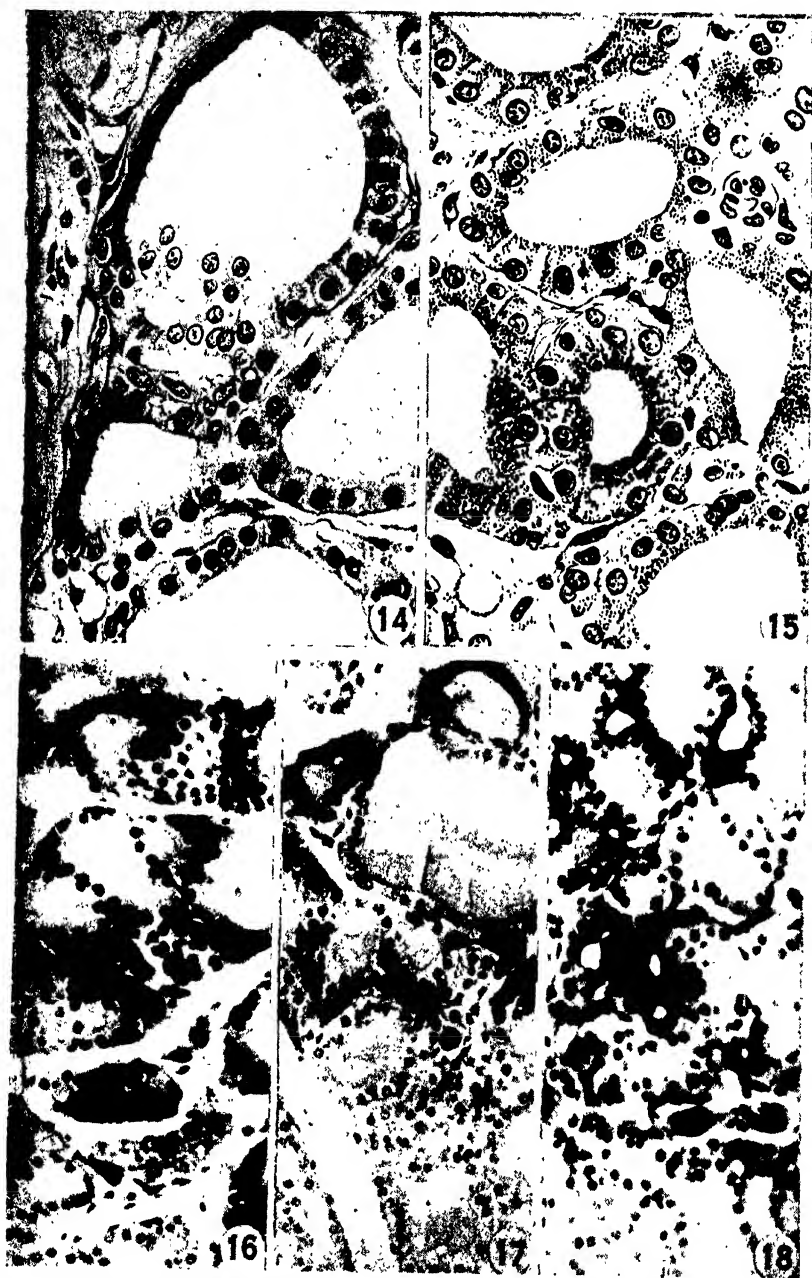
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### FIGURES 10-13 (See opposite page)

FIGURES 10 and 11. Argyrophilic granules in the cytoplasm of the thyroid cells from a normal rat (FIGURE 10) and a rat exposed to cold (FIGURE 11). The glands were fixed in Zenker's acetic mixture and stained by Pap's stain. The sections were drawn with a 90X objective and a 10X ocular.

FIGURE 12. Thyroid gland from a normal rat, illustrating the reaction of the colloid with the Schiff leucofuchsin reagent according to Bauer's method. X 200.

FIGURE 13. Section of the thyroid gland from a dog, illustrating the argyrophilic granules displayed in the follicular cells by Bodian's protargol method. 90X objective, 10X ocular.



The figures on this plate illustrate phosphatase reactions of the rat's thyroid gland.  
(For description see facing page)

follicular epithelium. The former enzyme characteristically is located as granular deposits in the apical portions of the more active follicular cells (FIGURE 15). In the rat, such cells occur in the central follicles, whereas toward the periphery of the gland larger follicles containing dense colloid and the flattened epithelium characteristic of storage are found. These peripheral follicles are devoid of acid glycerophosphatase, but contain moderate quantities of the alkaline enzyme (FIGURE 14). After incubation in other substrates, the epithelium of the peripheral follicles exhibits alkaline phosphatases which dephosphorylate adenylic acid, yeast nuclei acid, fructose diphosphate, and glucose-1-phosphate. These substrates, unlike glycerophosphate, are not acted upon by the rat thyroid at acid pH.

In addition to the phosphatases of the parenchymal cytoplasm, reactions are also obtainable in the nuclei of the follicular cells and in the endothelium of the capillaries. The nuclei react strongly at alkaline pH when nucleic acid or glucose-1-phosphate is used as substrate. The capillary endothelium exhibits some reactivity at alkaline pH with any of the substrates mentioned, but by far the most vigorous reactions are obtained with fructose diphosphate (FIGURE 16).

*The Demonstration of Iodine in the Thyroid Gland.* After the discovery that iodine was an essential component of the active principle of the thyroid gland, numerous attempts were made to locate this element in tissue sections. The earlier methods devised to distinguish iodine, which have been reviewed by Gersh and Steiglitz<sup>16</sup> and Lison,<sup>33</sup> have not yielded convincing results. More recently, three methods of more critical value have been employed. Turchini<sup>31</sup> determined the absorption of soft x-rays by sections of the thyroid gland. Strong absorption by the colloid occurred. Since the opacity of iodine to x-rays is well known, and since little ash which might contain other absorbing elements occurs in the colloid, it seems reasonable that the absorption observed by Turchini is a function of the iodine content. This promising method should receive further investigation. The second method involves the identification of radioactivity in the thyroid after the administration of tracer doses of radioactive iodine.<sup>25, 32</sup> By photographic methods, the radioiodine has definitely been located in the colloid, but the definition pos-

FIGURES 14-18 (See opposite page)

FIGURE 14. Alkaline glycerophosphatase (pH 9.4) reaction in the thyroid of a normal rat. The section was incubated in the substrate mixture for 6 hours. The reaction is confined to the peripheral part of the outer follicles. 40X objective, 7X ocular.

FIGURE 15. Drawing illustrating the location of acid glycerophosphatase (pH 4.7) in the thyroid gland of a rat exposed to cold. The reaction did not occur in the peripheral follicles. The section was incubated in the substrate mixture for 48 hours. 40X objective, 7X ocular.

FIGURE 16. Alkaline fructose diphosphatase (pH 9.5) reaction in the thyroid from a normal animal. The section was incubated in the substrate mixture for 24 hours. Note the concentration of enzyme in the endothelium. X 200.

FIGURE 17. Thyroid gland from a rat exposed to cold, illustrating the reduction in the alkaline fructose diphosphatase reaction. X 200.

FIGURE 18. Alkaline fructose diphosphatase in the thyroid of a rat to which thiouracil had been administered. X 200.

sible with the method is so slight that the amount of iodine present in the parenchymal cells cannot yet be determined. The third method depends upon the specific absorption of diiodotyrosine and thyroxine at 2800 Å. Gersh and Caspersson<sup>15</sup> found that colloid strongly absorbed ultraviolet light at 2800 Å, and demonstrated further that, within the follicular cytoplasm, vacuoles occurred, the absorption in which was identical with that of the intrafollicular colloid.

*Chemocytological Changes Associated with Different Phases of Thyroid Activity.* The preceding sections contain descriptions of several cytological or chemocytological reactions which differ in intensity in the peripheral and in the central follicles of the rat thyroid gland. The most reasonable interpretation of these changes is that the reactions of the central follicles are associated with active release of the thyroid hormone as compared with those of the peripheral units which are engaged in storage of the gland's active principle. Fortunately, more direct information on the state of activity comes from the study by Dempsey and Astwood<sup>8</sup> of the rate of secretion of the thyroid under different environmental conditions, in which it was shown that exposure to cold roughly doubled the rate of secretion, whereas maintenance in a hot environment greatly decreased the release of hormone into the systemic circulation. The cytological reactions described in the present account permit the conclusion that states of increased release of the stored hormone are accompanied by an increased height of the follicular cells, by a marked decrease in the concentration of the ribonucleoproteins responsible for the basophilia of the colloid, and by a slight decrease in the concentration of the nuclear desoxyribonucleoproteins of the follicular cells. In the colloid, concentration of reducing substances is decreased during active release of the secretion, as is also the intensity of its autofluorescence. The sudanophile droplets of the follicular epithelium increase in active states. In the capillary endothelium, the alkaline phosphatase reactions decrease in intensity (figures 16 and 17), whereas in the follicular epithelium acid glycerophosphatase puts in its appearance. On the other hand, in storage or inactive states, these reactions are altered in the reverse direction.

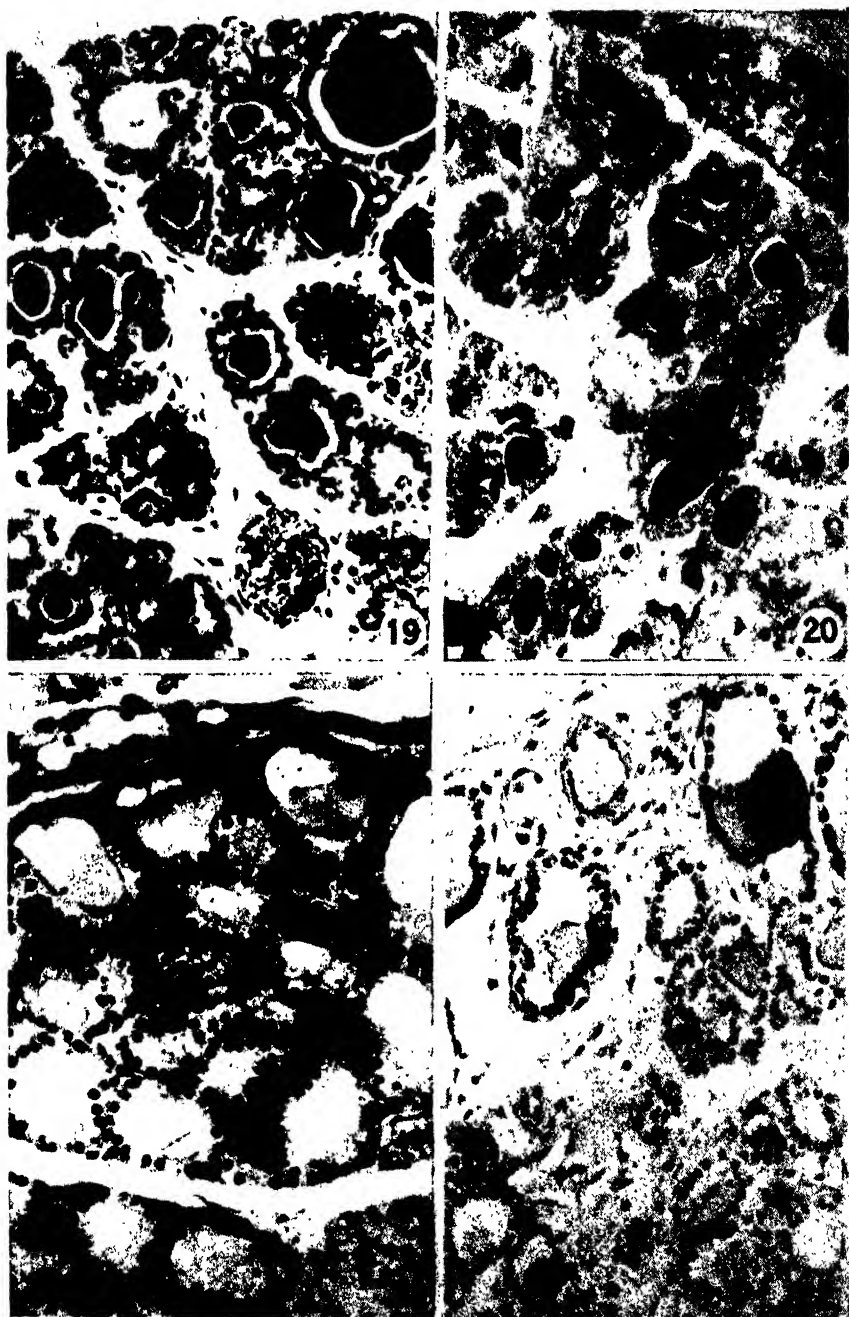
Although the experiments involving maintenance of rats in hot or cold environments are helpful in determining the cytological correlates of increased release of the thyroid hormone, they unfortunately do not illuminate the similar correlates of synthesis or manufacture of the active principle. In other words, there is no information bearing upon the rate of hormone synthesis in the cold-stimulated animal. This difficulty can be partly overcome, however, by a different type of experiment. MacKenzie and MacKenzie<sup>34</sup> and Astwood, Sullivan, Bissel, and Tyslowitz<sup>3</sup> have shown that various antithyroid drugs prevent the formation of thyroid hormone, profoundly alter the uptake of iodine by the gland,<sup>2</sup> and lead to a rapid exhaustion of the stored material. The pres-

ent account indicates that poisoning with thiouracil causes a series of cytological changes in the gland. The more prominent of these are an increased height of the follicular cells, a decrease in the nucleoproteins of the cytoplasm, colloid and nuclei, a decrease in the reducing substances and in the autofluorescence of the colloid, an increase in the number of sudanophile droplets in the epithelium, and an increase in the alkaline phosphatase reactions of the capillaries (FIGURE 18). Comparison of this list with the similar one in the preceding paragraph indicates that they differ in only one particular, namely, that the endothelial phosphatase is decreased during active release of the hormone but is increased after hormone synthesis is blocked. The two lists are derived from similar situations in that active release of stored hormone characterizes both, but are different in that hormone synthesis is blocked in the second and is normal in the first. It would seem, therefore, that, of all the changes listed, only the amount of endothelial phosphatase is related to the rate of synthesis of the hormone.

The cytological counterparts of synthesis of hormone can also be studied in a slightly more complicated experimental procedure. It has been shown previously that the normal store of thyroid colloid can be completely exhausted by the administration of thiouracil for a sufficiently long period. If animals in which such depletion of colloid has occurred are hypophysectomized, colloid storage again occurs,<sup>3</sup> whether or not thiouracil administration is continued postoperatively. The colloid formed and stored under such conditions is abnormal in that it does not contain iodine.<sup>7</sup> Consequently, in the glands from animals maintained in hot environments, the phenomena of storage and synthesis occur, whereas, in the thyroids from rats given thiouracil and later hypophysectomized, one observes the phenomenon of storage of colloid without synthesis of hormone. The question, therefore, arises as to the cytological differences which may be observed in these two experimental situations.

In the "inactive" glands of animals maintained in a hot room, the follicular epithelium is low, the basophilic staining of both cells and colloid is intense, the reducing activity of the colloid is high, and considerable amounts of alkaline fructose diphosphatase occur in the endothelial cells. In the glands from rats treated with thiouracil to exhaust the colloid stores and subsequently hypophysectomized, the epithelium is also low, the basophilia of the cells and colloid is intense (FIGURE 19), the reducing activity of the colloid is high (FIGURE 20), but the phosphatase reaction of the capillaries is entirely absent (FIGURE 22). It appears, therefore, that the gland in which synthesis of hormone is occurring contains phosphatase, whereas the thyroid in which synthesis is blocked does not.

The results cited in the preceding paragraphs reveal an interesting but baffling relationship between the endothelial phosphatase and the activity of the thyroid. Phosphatase cannot be directly related to hor-



FIGURES 19-22 (*For description see facing page*)

mone synthesis, since the enzyme is elevated after thiouracil and depressed after hypophysectomy, yet synthesis does not occur in either case. Similarly, phosphatase is increased in concentration after thiouracil but depressed on exposure to cold, yet release of stored colloid characterizes both states. The enzyme is elevated on exposure to heat and depressed after hypophysectomy, although an increase in the amount of stored colloid occurs in both situations. All that can be said, therefore, is that phosphatase activity is altered in different physiological states, but in a fashion which defies correlation with the known physiological activities of the gland.

The cytological observations presented in this account indicate that an unsuspected number of products are secreted by the thyroid gland. It appears that the highly basophilic colloid which occurs during phases of storage owes its staining properties to the presence of nucleoprotein. The reducing activity of the colloid is not correlated with its hormone content, and, since reducing activity is not a characteristic of known ribonucleoproteins, presumably indicates the presence of an additional compound in the colloid. The fact that these two components continue to be formed in hypophysectomized rats to which thiouracil is administered indicates an ability of the gland to carry on complex syntheses even after poisoning with antithyroid drugs. Likewise, the active hyperplasia and hypertrophy of the gland after treatment with thiouracil indicate the ability of the thyroid cells to reduplicate rapidly their own protoplasm in a similar poisoned state. It seems likely, in view of these facts, that antithyroid drugs act at a point near the end of the metabolic chain of events leading toward hormone synthesis, rather than near the beginning where reactions supplying the materials for general cellular metabolism occur.

### *Summary*

The cytoplasm of the follicular cells and the intrafollicular colloid exhibit basophilia after staining with methylene blue. This basophilic staining presumably indicates the presence of nucleoprotein, since it is abolished by digestion of the sections in crystalline ribonuclease and since its behavior in buffered solutions of dye is similar to that of other regions in which ribonucleoprotein is a known constituent. On the other hand, the basophilia of the colloid presumably is not caused by sulfate-containing mucopolysaccharides, since it does not stain metachromati-

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(See opposite page)

FIGURE 19. Basophilia of the colloid regenerated after hypophysectomy. Thiouracil was administered to the animal until the colloid was completely exhausted; the rat was then hypophysectomized and the colloid allowed to regenerate. Zenker-acetic fixation, eosin-methylene blue stain. X 200.

FIGURE 20. Bauer reaction of the colloid regenerated after hypophysectomy. Bouin fixation, Bauer's stain. X 200.

FIGURE 21. Alkaline fructose diphosphatase reaction in the thyroid from a normal rat. Compare with FIGURE 22. X 200.

FIGURE 22. Abolition of the alkaline fructose diphosphatase reaction in the thyroid from a hypophysectomized rat. X 200.



cally with toluidin blue and since its behavior in buffered solutions of dye is unlike that of mucus and mast-cell granules in which mucopolysaccharides are a prominent part.

Argyrophilic granules may be demonstrated in the follicular cells by Bodian's method and by Pap's stain after Zenker fixation. Argyrophilia of the colloid, coupled with positive reactions with Bauer's leucofuchsin method, indicates the presence of a substance containing active carbonyl groups.

Phosphatase reactions are exhibited by the nuclei and cytoplasm of the follicular cells and by the endothelial cells of the thyroid capillaries. Acid glycerophosphatase is present only in the central, active follicles, whereas several alkaline phosphatases occur in the peripheral follicles and in the endothelium.

The various reactions listed above are altered during different states of physiological activity. Alkaline phosphatase disappears after hypophysectomy. Basophilia and reducing activity are depressed during states of active release of the colloid, but may be formed in the hypophysectomized animal under conditions which prevent thyroid hormone synthesis.

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# THE METABOLIC CIRCUIT OF THE THYROID HORMONE\*

By WILLIAM T. SALTER

*Laboratories of Pharmacology and Toxicology, Yale University School of Medicine,  
New Haven, Connecticut*

IT is the purpose of this communication to emphasize the importance of the extra-thyroidal iodine in the body and particularly the pathways of distribution of thyroid hormone after its release from the parent gland. Intimately related to this problem is the fate of endogenous iodide released by the decomposition of the hormone which is degraded through use in the peripheral tissues. The iodide in the body is in constant circulation. As thyroid hormone is destroyed by metabolic processes in tissues, much of its inherent iodine can be recovered by the thyroid gland and reconstituted into active hormone.<sup>1</sup>

What is the mechanism by which the gland is able to trap iodide and concentrate it from very dilute plasma? What are the mechanisms by which the hormone is conveyed from the parent gland to various organs and individual cells? These are problems which have been neglected because general interest has been attracted to, and focused upon, the thyroid gland itself.

*The Conveyance of Thyroid Hormone to Body Tissues.* It is not yet settled how the hormone is delivered from the gland into the bloodstream. It seems clear that, under certain circumstances, thyroglobulin can appear both in the blood and in the lymphatics leading directly from the gland.<sup>2,3</sup> It seems likely, however, that the appearance of thyroglobulin in the bloodstream is the result of trauma or inflammation in the gland and cannot be regarded as indicative of a normal physiological process. Likewise, the demonstration by immune reactions of thyroglobulin in lymphatics may be a simple reflection of experimental trauma. At the moment, therefore, we must evade this question for lack of suitable experimental evidence and take up the trail of the hormone after it reaches the blood.

The distribution of the hormone in the blood involves two questions. First, do the red cells carry any hormone? Second, with what plasma constituents is the extracellular hormone associated? With respect to both of these problems, there are still dissenting opinions, but most investigators agree that the hormone usually is precipitable along with

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For the thyroxine used we are indebted to E. R. Squibb & Sons.

For the thiouracil used we are indebted to Calco Chemical Division, American Cyanamid Company.

protein.<sup>4, 5</sup> Therefore, a simple coagulum of plasma protein or of the mixed proteins of laked blood will carry down all of the circulating, organically bound iodine under ordinary circumstances. Such precipitates, however, are notoriously prone to carry down smaller molecules by adsorption,<sup>6</sup> so that special precautions must be taken in acquiring pertinent data and scrutinizing such evidence.

With regard to the hormone in red cells, the best thing that can be said at the moment is that the erythrocytes should be avoided. Certainly, it is clear that the use of plasma alone is desirable both technically and theoretically because it is in direct equilibrium with the tissue fluids. Data from two excellent sources<sup>7, 8</sup> are completely in disagreement, however, on the question as to whether the red cells contain "hormonal" iodine. One group of investigators reports that the concentration in red cells is approximately that in the plasma, while another finds no evidence of a "hormonal" iodine beyond that in the plasma. It would appear that much careful work must be done on this subject, which may require more refined methods than those available at present. For example, it is difficult to separate red cells cleanly from plasma without contamination. Furthermore, the precipitation of the proteins of laked blood by heat coagulation is difficult, inasmuch as the overlapping isoelectric points of various proteins tend to interfere with complete precipitation, so that the several protein fractions must be removed *seriatim*.

There is no question, nevertheless, but that the red cells may accumulate considerable concentrations of iodide ions, especially when iodide is administered to the organism in excess. Since the pioneer work of Wallace and Brodie,<sup>9, 10</sup> this fact has been confirmed repeatedly. In general, the distribution of iodide between cells and plasma is similar to the distribution of the chloride ion. Normally, however, the concentration of iodide in both erythrocytes and plasma is less than 1 microgram per 100 milliliters of water. It does not, therefore, present an analytical problem of importance except when pharmacological responses are involved.

*The Iodine in Plasma.* As to the plasma "hormonal" iodine, considerable data have now accumulated.<sup>11</sup> It is well established, in man, that the concentration of the circulating "hormonal" iodine is a good index of the physiological status of the organism with respect to thyroid activity.<sup>12, 13</sup> Even certain cases of apparent Graves' disease can be shown not to be hyperthyroid by this technical procedure, which agrees with the best clinical appraisal performed by a well-trained physician.<sup>14</sup>

In recent years, it has become apparent, furthermore, that the circulating "hormonal" iodine is indeed closely related to the plasma proteins. In particular, it has been possible to isolate most of the iodine-containing material in association with one or more of the main protein fractions.<sup>15</sup> In the early reports by the present author, the material

seemed to be associated with the smaller albumin fractions, at least in the major part.<sup>11</sup> When the plasma protein fractions of Professor E. J. Cohn became available, this point was pursued further and a somewhat higher concentration-peak was located in the alpha-beta-globulin fraction.<sup>16</sup> This latter fraction, however, although higher in specific concentration, did not account for a great mass of the total organic iodine. As the matter stands to date, the major single portion of the organically-bound iodine in the plasma resides with the albumin. The problem is under further analysis to see what the significance may be of the iodine which resides in the Fraction IV-6 of Cohn,<sup>17</sup> which is one of the smaller globulins.

For the moment, it might be said that these protein fractions are the sort that are expected to traverse capillary walls most readily. In the present communication, therefore, this would be the most significant finding to be emphasized with regard to these high-iodine plasma fractions. It is most likely that they are organically-bound iodine fractions, incorporated in the protein molecule. It is also conceivable, however, that one of them is free thyroxine, whereas the other fraction is incorporated into protein. These are details awaiting solution as better methods become available for handling smaller and smaller amounts of material. The analytical difficulty will be appreciated if it is realized that the most concentrated protein fractions yet obtained contain only three parts in a million. A representative series of analyses of these proteins is shown in TABLE 1. These are by no means the final results, but they indicate

TABLE 1  
IODINE IN PLASMA PROTEIN

<i>Protein</i>	<i>Fraction after Cohn</i>	<i>Protein, grams per liter</i>	<i>Iodine, <math>\mu</math>g. per liter</i>	<i>Iodine, <math>\mu</math>g./100 gm. protein</i>
Albumin				
small	VI	31.3	28.7	92
large	V	20.0	9.4	4.7
Globulin				
alpha	IV-2	0.7	1.2	193
beta	IV-1	1.3	1.7	139
gamma	II+III	18.5	9.0	41
Fibrinogen	I	3.6	1.95	55
<i>Total</i>		<u>75.4</u>	<u>52.0</u>	<u>524.7</u>

the nature of the problem and the experimental approach which is being pursued.

It will be observed, from a perusal of the fractions involved, that these might be expected to penetrate into the tissue fluid and ultimately to be collected by the lymphatics draining the tissue spaces. It is difficult, of course, to analyze pure tissue fluid, but, by comparing the content of the plasma and of the lymph simultaneously, one is able to bracket the problem and so to discover what are the probable intermediary values. It will, therefore, be interesting to pursue the problem further into body fluids

in order to see what sort of relationship exists between the iodide and the protein-bound iodine of these fluids as related to the plasma from which the fluids are derived.

**Protein and Iodine in Various Body Fluids.** The iodide can be disposed of at once. Ordinarily, it is very low in concentration and constitutes a relatively trivial problem. It is not surprising to find concentrations of 0.5 microgram per cent in such fluids as pericardial fluid, spinal fluid, cervical lymph, and lymph from the extremities. Of course, under pharmacological circumstances, the concentration may be much higher. For example, when large doses of iodide such as used to be administered in tertiary lues are given, the plasma iodide in man may mount to concentrations of 1 or 2 milligrams per cent<sup>4</sup> and then fall off over the course of many hours toward the normal. Even an overnight fasting blood may show values above 100 micrograms per cent if a large dose of iodide has been administered on the preceding day. If the plasma protein is precipitated in such a medium, it will carry down with it a high concentration of adsorbed iodide ions and thus give a false impression of high hormonal iodine, as shown in FIGURE 1. Under such circumstances, a dialysis

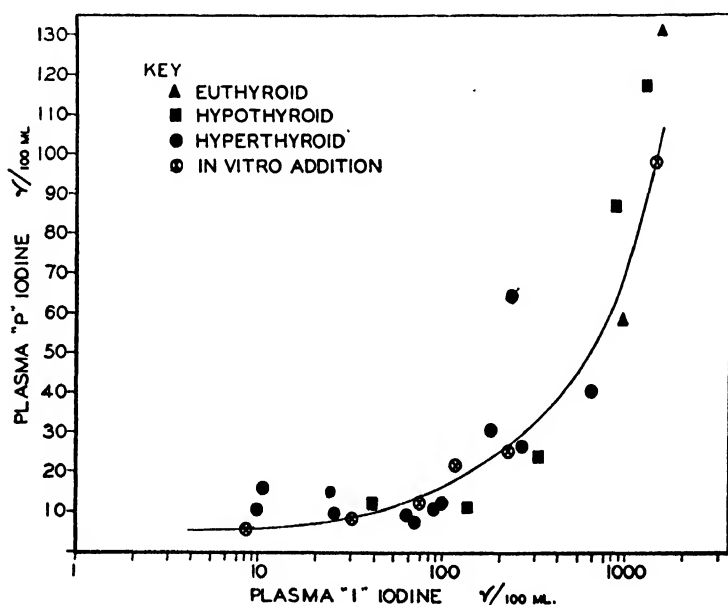


FIGURE 1. In the presence of unusually high concentrations of serum iodide (inorganic), adsorption of iodide ions upon the protein precipitate may yield spuriously high results for apparent "hormonal" iodine. (From BASSETT, COONS, & SALTER,<sup>5</sup> p. 522.)

or some other procedure is necessary to rid the plasma protein of the tremendous excess of iodide ion before analysis of the protein.<sup>6, 18</sup>

The same sort of situation may obtain when iodide is introduced into the body by other means. For example, if the sodium salt of mono-iodo-

methane sulfonic acid ( $\text{CH}_2\text{I}.\text{SO}_3\text{Na}$ ), which contains 52 per cent iodine, is injected intrathecally, this material serves as a reservoir for iodine which will pay back into the plasma over long periods. With the collaboration of Dr. Donald Munro of the Boston City Hospital, this problem has been studied in our laboratory.<sup>19</sup> Under these circumstances, as shown in TABLE 2, values of plasma iodine may rise as high as four milli-

TABLE 2  
TOTAL IODINE OF CEREBROSPINAL FLUID AND OF SERUM  
AFTER INTRATHECAL ADMINISTRATION OF  
SODIUM MONO-iodo-METHANE-SULFONATE  
(Both concentrations are expressed in milligrams per cent.)\*

Patient	0		$\frac{1}{2}$		1		2		3		18		24	
	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum	Fluid	Serum
He	0.5				11.6									
Ya	0.0				11.1		2.8							
Whe	0.0				10.0		1.6							
Elk	0.0				9.6		5.5							
Har	0.0				6.0		3.2							
Ra	0.0				5.0		3.9							
En	0.0				4.6		2.5							
Wa		0.0		0.5			4.2				10.8		0.6	
Pi			23.5		4.5	.3	4.2	0.4	2.6	0.3				
Cr				1.9	10.3	0.7	3.1	1.1					0.1	
Wal		0.04		3.3		4.3		2.7						
DeF		0.02		2.7		3.4		2.7						
Chi		0.07		1.3		1.6		1.1						
La		0.01		1.1		1.9		2.9						
Pa		0.3		0.3		1.5		1.6						

\* Excerpt from data of a study in collaboration with Dr. Donald Munro of Boston. The author wishes to thank the Sterling-Winthrop Chemical Company for the sample of "Skiodan" used.

grams per cent within a few hours after administration. A comparison of FIGURES 2 and 3 will indicate how rapidly the exchange occurs. When the plasma so obtained is fractionated, it would appear that part of this material exists in the form of free iodide ion. However, the compound in question is supposed to undergo ionization. Therefore, on extraction with *n*-butyl alcohol, some of the iodine accumulates in the organic phase. In fact, the author obtained approximately even distribution of the material between alcohol and water. This finding suggested that at least half of the excessive iodine-containing material found in plasma under such conditions is not simple iodide but associated with an organic carrier, presumably the original "Skiodan,"<sup>20</sup>  $\text{CH}_2\text{I}.\text{SO}_3\text{Na}$ .

In short, for smaller iodine-containing molecules there is a constant equilibrium across body membranes, or rather a tendency to equilibrium. This tendency to equilibrium is disturbed by kinetic factors so that pure thermodynamic equilibrium probably never is completely attained. For

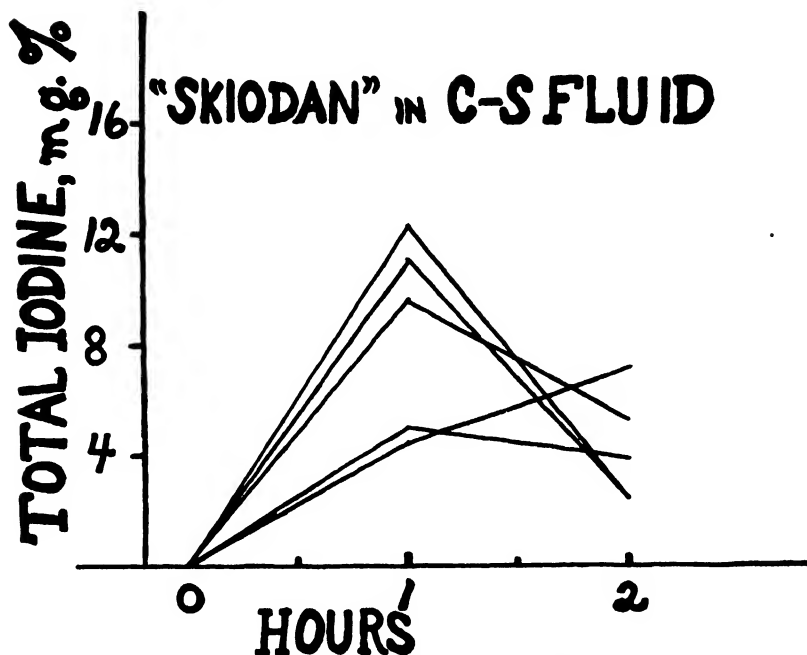


FIGURE 2. Total iodine in human cerebrospinal fluid after intrathecal instillation of sodium salt of mono-iodo-methane sulfonic acid.

practical purposes, however, one can use the thermodynamic equilibrium as a hypothetical picture toward which the fluids tend to move under actual circumstances. When the spinal fluid, to cite another example, is examined after the administration of high doses of iodide by mouth or intravenously, it is found that the concentration of iodide rises after a certain lag and, furthermore, fails to achieve as high a peak as the plasma.<sup>21, 22</sup>

The problem is complicated further by the fact that many organic compounds which contain iodine are constantly undergoing degradation into their constituent iodide. For example, when diiodotyrosine is administered intravenously or by mouth, it begins at once to decompose into iodide.<sup>23</sup> Therefore, it is questionable whether the thyroid gland itself can ever be treated by diiodotyrosine alone, except by perfusion experiments upon the isolated gland.<sup>24</sup>

Such dynamic considerations, however, are of minor quantitative significance, as already indicated. The important point is that throughout the body there tends to be an approximate equilibrium between the tissue fluids, provided free exchange of the iodine compounds can be achieved. Consequently, the fact that the hormone is anchored to protein carriers may be of considerable importance because, in this way, its distribution and consumption by tissue cells can be regulated. In view of the marked stimulating effect which the thyroid hormone has upon a



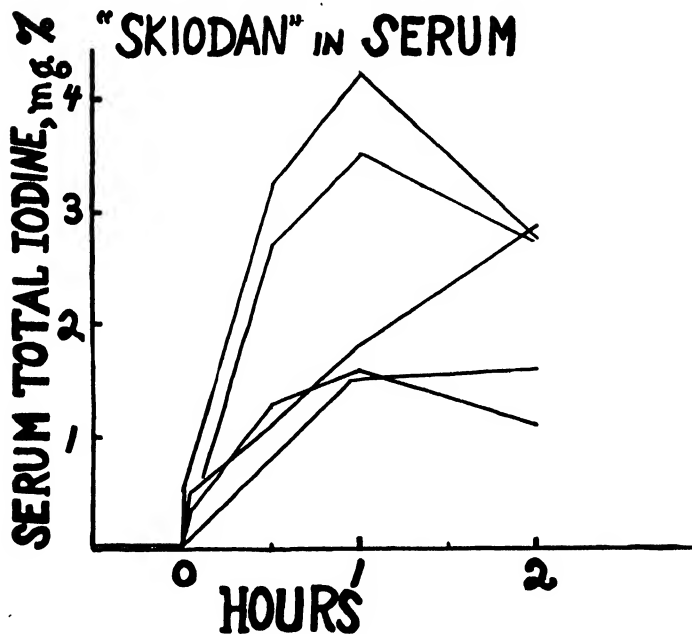


FIGURE 3. Total iodine in human blood serum after intrathecal instillation of sodium salt of mono-iodo-methane sulfonic acid. Excerpt from data obtained in collaboration with Dr. Donald Munro.

wide variety of metabolic processes, it may be a very desirable feature of its control that this very active hormone, which affects so many bodily processes, is anchored effectively to slow-moving colloidal molecules.

Through the kindness of Professor Cecil K. Drinker,<sup>25</sup> the author and his associates were able to obtain samples of various tissue fluids in the dog.<sup>16</sup> Because, in general, the dog operates at a lower concentration of "hormonal" iodine than does man, the data in question have been divided by a factor of 0.71 in order to make them comparable to human values. As shown in TABLE 3, in these animals the plasma protein and plasma iodine (including both the "hormonal" and inorganic fractions) were determined simultaneously. Out of these observations come several interesting features:

- (1) The "hormonal" iodine of the body fluids is lower than that of the plasma.
- (2) The protein of the body fluids is lower than that of the plasma.
- (3) There is relatively more iodine in the clear fluids than their total protein content would indicate on a proportionate basis. In other words, the higher iodine-containing fractions of the plasma proteins apparently have leaked through the capillary wall preferentially.
- (4) The iodine concentration of the body fluid is low and approximately that of the plasma.

If one attempts to correlate these findings, one is led to the conclusion, as a first working hypothesis, that the "hormonal" iodine is associated with those protein fractions which can more readily leak through the capillary walls into the perivascular tissue spaces. After this body of water has laved the tissue cells, it is then drained into the lymphatics, with the results shown in TABLE 3.

TABLE 3  
PROTEIN-BOUND IODINE IN LYMPH\*

Dog No.	3 M	4 M	5 M	6 M	7 F
<i>Serum</i>					
Protein, gm. %	8.3	8.0	7.5		
P-iodine, $\mu$ g. %	4.6	9.8	4.3		6.2
<i>Pericardial fluid</i>					
Protein, gm. %	1.6				
P-iodine, $\mu$ g. %	1.3				
<i>Rt. cervical lymph</i>					
Protein, gm. %	2.6	3.0		3.3	
P-iodine, $\mu$ g. %	2.6	5.1		5.3	4.4
<i>Left cervical lymph</i>					
Protein, gm. %	2.6		2.6 hours	3.1	
P-iodine, $\mu$ g. %	2.8		3.9 hours later	3.8	4.0
<i>Left forepaw</i>					
Protein, gm. %				2.2	
P-iodine, $\mu$ g. %				5.7	

\* For convenience, the data for iodine concentration have been divided by the factor 0.71 in order to step up canine values to human levels.

A special case is that of the thoracic duct. This is one of the largest lymph vessels in the body. The natural tendency of the investigator is to rush to this main trunk. Unfortunately, the material which it drains comes from the gastrointestinal tract and may contain very large amounts of iodine, mostly inorganic. The results, therefore, are confusing. [Dr. Drinker has referred to this fluid privately as "bilge"!]. The author agrees that one should be very cautious in interpreting the results of iodine analyses attained in this fluid, if there is any chance at all that absorption from the gastrointestinal tract may be in progress. This situation is somewhat similar to that of the bile as reported by Krayer<sup>26</sup> and by Zawadowsky and Perlmutter.<sup>27</sup> If large amounts of thyroid hormone are given intravenously or even fed to dogs over a short period of time, the thyroid iodine spills into the bile. Indeed, it may be excreted so rapidly that it may even escape destruction by the liver and actually may be recognizable as potent hormone in the biliary excretion. Obviously, such findings constitute a special case which might be called "detoxification" and should not be classed with concentrations which represent a working equilibrium between various organs.

There is still much to be learned concerning the means by which the thyroid hormone is associated with the plasma fractions. Thyroxine, being an amino acid, conceivably may be built into the body of the cir-

culating protein just as any other amino acid is so constituted. Indeed, the work of Schoenheimer<sup>28</sup> and his school has indicated that there is a constant flux of metabolites in the organism, so that any single measurement reflects merely the status of the so-called "metabolic pool" at that particular instant. There is also the possibility, raised by Cohn,<sup>29</sup> that hormones and enzymes are carried in the plasma fractions by some sort of co-valent bonds. These co-valent bonds would be sufficiently strong to prevent removal by ordinary dialysis and perhaps to prevent separation in certain organic solvents. Such an association, however, would not necessarily imply a peptide linkage. These are problems which will be settled more readily when the work which is going on in collaboration with Dr. Cohn's laboratory has been extended further.

*The Iodine in Tissues.* There are two general problems concerning the regulation of the iodine in tissues. The first concerns the entrance of iodide; the second, the admission of "hormonal" iodine. Furthermore, the problem must be subdivided with respect to specific tissues involved. Obviously, those tissues which have a specific mechanism for trapping iodide—presumably by the formation of a new chemical substance—will react somewhat differently than those tissues which have no such specific iodine-bearer.

With regard to the inorganic fraction, the distribution of iodide in body tissues at large seems to have been well summarized by Wallace and Brodie,<sup>9</sup> who showed that the general distribution was rather similar to that of the chloride ion. They demonstrated, for example, that the ratio of such ions in plasma water and in tissue water was approximately the same for both chlorides and iodides. The principal exception was brain tissue, in which chloride seemed deficient relative to plasma.<sup>10</sup> The great exception to this general picture, obviously, is the thyroid gland itself. It still remains a considerable puzzle how the thyroid is able to trap iodine from such low concentrations as exist in the plasma. Nevertheless, certain suggestive results have been obtained in the author's laboratory in rats treated with thiouracil over the course of weeks and months. In a previous communication,<sup>30</sup> it was shown that, when iodide was given together with thiouracil in high concentration, the glands of such animals contained as much iodine as a normal gland. Of course, the concentration was lower than normal because the gland had increased in bulk through hypertrophy and hyperplasia. When such glands were fractionated, it appeared at first sight that most of the iodine was apparently "inorganic" in type. This result has been reported from a number of laboratories.<sup>31, 32</sup>

The author, however, raised the question whether this "iodide," so-called, may not indicate an intracellular enzymic mechanism at work within the hypertrophied tissues. Accordingly, after 8 months of treatment with these thyroid blocking agents, the animals' glands were removed, extracted, and analyzed after certain chemical preliminary pro-

## SPECIMEN PROTOCOL FOR TABLE 4

Group B rats received a standard dog chow such that 16 grams contained 13 micrograms of iodine. This was the approximate daily ration which the animals consumed during the earlier part of the experiment.

Group G rats were fed the same chow reinforced with thiouracil and potassium iodide. The formula for the diet was

ground dog chow	983.6 gm.
thiouracil	10.0 gm.
KI	6.4 gm.
	<hr/> 1,000.0 gm.

Group F rats were fed chow reinforced with potassium thiocyanate and potassium iodide. The formula for the diet was

ground dog chow	973.6 gm.
KSCN	20.0 gm.
KI	6.4 gm.
	<hr/> 1,000.0 gm.

The protocol for rat no. 25G follows:

This male rat was started on the diet containing thiouracil and potassium iodide on April 26, 1945. At that time, he weighed 250 grams. He was autopsied on March 27, 1946, at which time he weighed 383 grams. The body length from nose to breech was almost exactly 9 inches. The tail was 8 inches long. The pituitary weighed 15.7 milligrams; the thyroid 298.8 milligrams. One lobe of the thyroid was preserved in the formalin fixative at the same time that the pituitary was preserved in a Zenker fixative.

The other lobe of the thyroid, weighing 157.2 milligrams, was homogenized in dilute ammonia and the resulting suspension diluted to 4.0 cc. Each of the four 1-ml. aliquots was used for iodine determinations as indicated in the table. The total iodine was determined, the iodine after dialysis for twenty-four hours, and the heat-coagulable iodine. The last aliquot was brought to pH 6.0 and treated with 15 volumes of acetone. After standing for twenty-four hours, the resulting protein precipitate was separated, and the iodine determined both in the precipitate and in the supernatant fluid. The results are indicated in TABLE 4.

Simultaneous determinations were made of the iodine in skeletal muscle from the thigh region. In some samples, the total iodine content was determined directly. Other samples were homogenized, subjected to heat coagulation, and the respective iodine contents in the coagulum and the supernatant fluid determined separately.

cedures. Some of the pertinent data are given in TABLE 4. It will be observed that, if the extracted gland substance is subjected to heat coagulation, much of the iodine can be removed by this procedure. These conclusions, at first sight, might lead to the assumption that the iodine consisted simply of dissolved iodide. Certain other considerations, however, open up the possibility that something more complex is occurring. The central feature of these experiments is the indication that the "iodide" is bound to some albuminous constituent of the cell or gland. For example, if iodide is added to a homogenate containing the same amount of extract from the normal glands, it is practically all lost in the course of 24 hours under certain conditions of dialysis. If, on the contrary, the same amounts of thyroid homogenate and of iodine from such a hypertrophied gland are selected in one sample and treated similarly, something like half of their iodine remains within the dialyzing membrane after the same period of time.

This finding suggests that there is present, in these hypertrophied

TABLE 4

## IODINE FRACTIONS IN FRESH THYROIDS OF MALE RATS TREATED WITH GOITROGENS

Rat No.	Wt. of rat, gm.	Wt. of thyroid, mg.	Fraction of homogenate	Iodine in thyroid (micrograms)			
				Total iodine	After dialysis	Heat coagulable	P'pt'd by acetone
14 B							
Control	270 to 327*	32.0	Protein Iodide		0.3	0.7	0
			Sum	2.7		2.5	3.0
21 G							
Thiouracil plus KI	220 to 296	167.1	Protein Iodide		2.0	2.7	4.7
			Sum	11.5		8.3	6.9
25 G							
Thiouracil plus KI	250 to 383	298.8	Protein Iodide		3.2	3.0	6.1
			Sum	14.0		9.6	7.9
22 E							
Thiocyanate plus KI	290 to 376	35.9	Protein Iodide		1.5	3.5	0
			Sum	3.3		2.4	4.1

\* Weights are given for the beginning and the end of the eight-month period during which the animals were under study.

glands, an expanded biochemical system which has a high affinity for iodine. A similar result is achieved when, instead of dialyzing and heat coagulating, one subjects the extract of the hypertrophied thyroid to precipitation by acetone at pH 6. Under these circumstances, it is possible to precipitate iodine-containing protein material, as shown in TABLE 4. When normal thyroid tissue is treated similarly, on the other hand, the amount of iodine-containing protein so precipitated is almost negligible. At present, these experiments can be regarded only as suggestive because there may be differences in the relative water and protein contents of the normal and the hypertrophied glands, respectively, which might affect their physico-chemical behavior. Perhaps, also, the thiouracil present may serve to mordant the iodine to the colloidal carrier.

Therefore, the suggestion should merely be raised that, when hyperactivity is induced in a thyroid by thiouracil, there occurs concomitantly with visible cellular proliferation a hypertrophy of the constituent enzyme systems. These so-called iodases are concerned with the trapping of iodine and its subsequent synthesis into diiodotyrosine. This increase in the mass of intracellular enzyme makes available a

larger amount of colloidal carrier upon which iodide ions can be trapped. In this way, when iodide arrives inside the follicular cells and conceivably also within the thyroid follicle, a part of the dissolved iodine will become associated with colloid carriers and part of it will remain free. Indeed, in connection with the titration curves of proteins and the calculated acid-binding capacity thereof, it is a routine procedure for physical chemists to correct the amount of acid added for the free or ionized portion of the anion.<sup>33</sup> In other words, in the titration of a protein with hydriodic acid, due cognizance would routinely be taken of "free" and "bound" iodide, conveniently designated as fractions  $I_F$  and  $I_B$ , respectively. Obviously, such an enzyme complex, in order to be effective physiologically, would participate in a change in the state of oxidation of the iodide ion to a state which is equivalent to elementary iodine. It is conceivable, therefore, that such iodine-conveying systems may contain both an iodinase and a peroxidase component. This possibility arises by analogy with the well-known enzyme systems which convert molecular oxygen into oxygen available for biological oxidative processes.<sup>34</sup> Much more work obviously must be done along these lines before one can speak definitely. Keston<sup>35</sup> has offered an important model of this sort by using the xanthine oxidase of milk to form thyroxine. It is evident from TABLE 4 that thiocyanate prevents the initial trapping of iodide by the colloid, iodinase. Thiouracil, however, acts at a later stage in the iodine economy of the thyroid gland.

*The Trapping of Radio-Iodide by the Thyroid.* Radioactive iodine has been used for three main purposes in connection with the thyroid. In the early experiments, such as those of Hamilton and Soley<sup>36</sup> or Hertz, Roberts, and Evans,<sup>37</sup> labeled iodide was used. The results so obtained obviously indicated the effect and fate of the iodide carrier used, and were readily evaluated. With the advent of highly radioactive isotopes, however, the "tracer" dose has been used extensively. Unfortunately, it is uncommon to find in the literature simultaneous analyses of total iodine by classical microchemical methods. This second type of application of radio-iodide, therefore, has led to much confusion, because many investigators have failed to realize how important it is to determine *specific* rather than *absolute* radioactivity in tissues. Consequently, many of the "tracer dose" experiments cannot be interpreted in physiological terms. The problem is further complicated by the possibility of atomic interchange ("the exchange reaction"), as discussed by Salter.<sup>12</sup>

The third use of radio-iodide has been for destructive purposes, as described by Hertz and Roberts<sup>38</sup> and Chapman and Evans.<sup>39</sup> This application has led to a surprising effect on iodine balance, especially when applied to certain cases of thyroid neoplastic disease in which the total mass of metastatic tissue was large. In collaboration with Dr. F. Hamburger<sup>40</sup> of the Memorial Hospital, the author has had the opportunity of studying the effect of such therapy upon the circulating iodine. Only

two short excerpts from the data can be cited here. One patient, whose estimated metastatic tissue approximated two kilos, went into "thyroid storm" after the administration of highly radioactive iodide. Although the total energy so delivered was large, the amount of iodide administered was less than a microgram. Nevertheless, the patient's circulating "hormonal" iodine rose to 26 micrograms per cent and the inorganic fraction to 560 micrograms per cent. Obviously, by the dissolution of the several masses of thyroid tissue, excessive amounts of thyroxine and of iodide had been released into the circulation. Another patient showed temporary hyperthyroidism, followed by hypothyroidism, as destruction or inhibition of thyroid tissue approached completion. This reversal of thyroid status is illustrated by the short series of analyses in TABLE 5.

TABLE 5

SERUM IODINE VALUES AFTER TREATMENT WITH HIGHLY RADIOACTIVE IODIDE\*

	<i>Date</i>	<i>"Hormonal" iodine</i> <i>µg. per cent</i>	<i>Iodide</i> <i>µg. per cent</i>
Before treatment	12 3 46	4.7	1.6
After treatment	12-10-46	12.1	8.6
	12-17-46	10.4	2.4
	12 24-46	4.1	2.7
	12-31-46	3.8	2.0
	1-2-47	4.1	3.4
	1-7-47	6.3	7.0
	1-14-47	5.0	12.8
	1-21-47	4.1	1.8
	1-28-47	4.5	14.2

\* The arbitrary normal limits of "hormonal" iodine are considered to be 4.0 and 8.0 micrograms per cent.

*"Hormonal" Iodine in Tissues.* In considering the organically bound iodine in tissues, one must consider two specific cases. The first is the high concentration of iodine reported in certain endocrine glands, notably the pituitary and the ovary. This problem will be passed by in the present communication, except to note that Courrier<sup>41</sup> has reported that radio-diiodotyrosine is fixed specifically by the anterior pituitary of rabbits, whereas a similar amount of radioactive potassium iodide produces no such accumulation. Therefore, Courrier would agree with the older investigators who stated that the pituitary had the capacity for concentrating iodine and particularly for trapping thyroid hormone. The ovarian iodine, as reported by Perkin,<sup>42</sup> is also high in the presence of an actively functioning thyroid and tends to fall in myxedematous animals. It still remains to be shown whether the iodine in these glands is derived from prefabricated thyroid hormone or whether these glands themselves have some property of synthesizing an organic iodine complex. Moreover, the high lipid content of these organs tends to yield high false values for iodine. Therefore, further study by modern analytical techniques are needed before physiological conclusions can be drawn.

With regard to the second case, namely, that of iodine in the tissues at large, the problem seems to center primarily in skeletal muscle. It has long been known that the bulk of the body iodine is located in skeletal muscle and, exclusive of the thyroid, over three-quarters of it is so confined.<sup>43</sup> Many years ago, Kommerell<sup>44</sup> studied the effect of feeding flesh from normal dogs as against flesh from thyroidectomized dogs. He reported that the latter sort of meat produced no "specific dynamic action" of protein as expected. It still remains debatable what this finding signified, but other investigators since then have shown that after thyroidectomy the stores of iodine within the body decline.<sup>45</sup> Moreover, at the same time, the urinary excretion of iodine increases because no trapping mechanism is available for the reconversion of the iodide into hormone. If one is willing to assume that the extra iodine excreted in such metabolic studies represents degraded hormone, one can calculate the amount of hormone originally held in body muscle. It is now becoming possible to confirm such computations by the direct analysis of skeletal muscle.<sup>46</sup> In brief, it seems clear that, in frank myxedema, uncomplicated by large doses of exogenous iodine, there is very little iodine within the muscle. Indeed, the total iodine of the muscle approaches that to be predicted from the circulating iodide and is probably less than 1 microgram per cent. In fact, some analyses show bare traces of iodine, even when one uses analytical techniques adapted to the analysis of "trace" elements.

On the other hand, in animals made hyperthyroid by the administration of hormone of *exogenous* origin, the iodine in the peripheral tissues increases perceptibly.<sup>47</sup> In general, one may say that there is a direct relation between the concentration of circulating plasma hormonal iodine and that of the tissues. The real problem, therefore, is the quantitative one, namely—How does the muscle-fixed iodine vary with respect to the plasma-fixed iodine? The answer to this quantitative problem is plagued by several technical difficulties. First of all is the difficulty of analyzing very small amounts of material. (In the author's laboratory, it has been possible, however, to obtain satisfactory results in rats with amounts of muscle approximating 250 milligrams.) The second problem is that of separating inorganic from organic iodine. If the iodine of the environment and, in particular, of the ingesta is low, this problem is minimized. When iodide is administered, however, a major problem of analysis is involved, as shown in TABLE 6. It is especially difficult to rid tissue extracts of adsorbed iodide ions, and fantastic values for "hormone" iodine may be attained. The next difficulty is that of species. Indeed, McClendon<sup>48</sup> has suggested that in the smaller mammals which (as it were) live faster, the bound iodine of the peripheral tissues runs higher. He gives such values, for example, as 6 micrograms per cent in the steer as against 24 micrograms per cent in the mouse. There are, as yet, not enough data available to test this statement. It is true, however, that the dog and the rat have lower values for circulating plasma "hormonal"



TABLE 6

TOTAL IODINE CONCENTRATION IN FRESH RAT TISSUES  
(MICROGRAMS PER CENT  $\times 2$ )\*

Rat No. normal	Brain (hypothalamus)	Heart	Kidney	Liver	Muscle	Serum
1	11	15	8	9	8	3
2	6	10	9	18	6	
3	12	12	8	19	9	
4	13	11	9	12	8	
5	15	12	8	12	8	
6	10	11	10	14	9	
7		8	10	13	10	
8	13	12	6	10	8	
9	16	22	10	12	10	
10	13	26	17	(33)	16	
11		13	8	17	13	
12	11	23		18	11	
13	10	7	6	10	6	
14	8	15	13	10	9	
15	26	24	15	(33)	10	
Average	12.6 $\pm$ 1.3	14.0 $\pm$ 1.6	9.7 $\pm$ 0.9	13.4 $\pm$ 1.0	10.7 $\pm$ 0.7	
<i>Treated with tracer iodide</i>						
16	8	11	9	16	11	

\* The variation in these data is probably due more to analytical technique than to variation in animals. Note that these relative values are twice the actual findings.

iodine than does man. One must be prepared, therefore, to find variations in the tissue iodine.

There is another problem to be solved with respect to the localization of iodine within tissues: Does the thyroid hormone operate only at the cell membrane, or does it penetrate the cell to become an integral part of a tissue enzyme mechanism? Should one be content to express "hormonal" iodine concentrations in tissues in terms of the fresh tissue, or should one extract the fat and reduce the iodine content to dry weight or even to phosphorous content? What is the effect of varying water content in the tissues? These many factors will have to be controlled, one by one, before a definitive answer to the question of tissue iodine can be reached.

At the present time, the best one can do is to state that there are not enough data to confute the previously published conclusion that the concentration of "hormonal" iodine in tissues is a logarithmic function of the basal metabolic rate.<sup>11</sup> Such a conclusion, at present, is based solely upon maintenance levels of metabolism. By integrating the decay curve with respect to time, one arrives at the conclusion before stated.<sup>1</sup> It is difficult to believe that such a conclusion can be fundamentally wrong. Rather, it seems that the chief contribution of careful tissue analysis will be a more accurate evaluation of the constants involved in equations such as those suggested by Boothby and Baldes<sup>49</sup> many years ago.

There is still another problem, however, which is of fundamental im-

port. This is the actual state of the organically-bound iodine within peripheral tissues. At the moment, the author is struggling with the isolation of plasma fractions and only a few preliminary experiments have been undertaken with skeletal muscles, as shown in TABLE 6. It appears, however, that muscle iodine, like plasma iodine, can be divided into two fractions, the first inorganic, the second protein-bound. Furthermore, it is possible to separate the muscle sap into fractions which are respectively low and high in relative iodine content. Eventually, as these experiments are pursued further, it should be possible to gain a better conception of the function of the thyroid hormone in the peripheral cell. Already it has been shown that muscle, kidney, and liver contain colloidally-bound iodine. In extracts of muscle from man, cat, and rat the protein-bound iodine can be separated into several protein fractions of different iodine contents.<sup>60</sup> In myxedematous rats this colloidal iodine vanishes; in hyperthyroid rats it rises to values several times the normal range.<sup>61</sup> The colloidally-bound iodine does not behave like free thyroxine added to muscle extracts. Therefore, this colloidal material has been named "thyrenzyme," because it appears analogous to co-carboxylase in relation to thiamine.

### *Summary*

Throughout the mammalian body, there is a continuous circulation of iodine in two main forms, namely, as iodide and as thyroid hormone. Whether other forms of organically-bound iodine exist normally is unknown, but when such compounds are encountered under pharmacological circumstances they also circulate in a characteristic fashion. The rather free movement of these organic and inorganic types is controlled by specific mechanisms which fix them in characteristic sites. The inorganic iodine is trapped and concentrated in the thyroid gland after abstraction from plasma which usually is very poor in iodide. There is no definite evidence that any other tissue in the body can effect so marked a concentration. The thyroid hormone, in the second instance, is fixed in two general locations. The first of these is the doubtful preferential accumulation in the anterior pituitary and the ovary. The second is the fixation of the hormone in peripheral tissues, notably skeletal muscle.

The conveyance of the two types of iodine differs. Iodide is evenly distributed throughout all body fluids and tissues including the brain. The "hormonal" iodine, however, is associated with certain fractions of the plasma proteins. As these colloidal fractions filter through capillary walls, they reach the tissue spaces and leave the cells before being drained into the lymphatics. The nature of the protein complex in muscle and other peripheral tissues which bind "hormonal" iodine is still largely unknown. This combination, however, seems to be concerned with the activity of tissue enzymes. When the tissue hormone is decomposed through use, it yields up iodide—and the cycle starts afresh.

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*Discussion of the Paper*

DR. M. L. TAINTER (*Sterling-Winthrop Research Institute, Rensselaer, N. Y.*):

Dr. Salter has referred to results which have been observed after administration of Skiodan intraspinally. It should be pointed out that Skiodan is not ordinarily injected in this way, nor is the solution designed for this form of administration. It is hypertonic and may cause irritation which can best be minimized by the previous induction of spinal anesthesia.

In view of the obvious difficulties of using an agent which requires previous anesthetization of the structures to which it is to be applied, it would seem to be desirable to emphasize that Skiodan should be injected intraspinally only under special circumstances and where there are persuasive reasons why this procedure must be used. It is not a diagnostic procedure to be used routinely, and in all probability it should be restricted to those who are highly skilled in its administration, and to cases where no safer alternative is available.

STUDIES ON  
THE FORMATION OF ORGANICALLY-BOUND  
IODINE COMPOUNDS IN THE THYROID GLAND  
AND THEIR APPEARANCE IN PLASMA  
AS SHOWN BY THE USE OF RADIOACTIVE IODINE

BY I. L. CHAIKOFF AND ALVIN TAUROG  
*Division of Physiology, University of California Medical School,  
Berkeley, California*

*The Formation of Diiodotyrosine and Thyroxine by the Normal  
Thyroid Gland*

THE work of many investigators has made it clear that 90 per cent or more of the iodine in the normal thyroid gland is present in organic combination. This organic iodine, according to Harington,<sup>1</sup> can be completely accounted for by thyroxine and diiodotyrosine. These two compounds do not normally exist free in the gland but can be isolated after suitable hydrolysis of the thyroid protein.

The application of radioactive iodine as a labeling agent provided for the first time a convenient method for measuring rates of formation of diiodotyrosine and thyroxine by thyroid tissue.<sup>2</sup> By this means, molecules of diiodotyrosine and thyroxine can be "dated," thus permitting a distinction to be made between molecules that were newly formed and those that were present before the introduction of the isotope into the body.

The radioisotope of iodine,  $I^{131}$ , which has a half-life of 8 days, has proved most useful for biological studies. It has been prepared by deuterium bombardment of tellurium in the cyclotron<sup>3</sup> and more recently has been obtained as a fission product of  $U^{235}$ .<sup>4</sup> Other radioactive isotopes of iodine ( $I^{128}$ ,  $I^{130}$ ), because of their short half-lives, are less useful for metabolic studies.

The present section deals with experiments in which the formation of diiodotyrosine and thyroxine by the thyroid gland of the intact animal was studied with the aid of  $I^{131}$ , which will hereafter be referred to as  $I^*$ .

*The Rate at which Administered Inorganic Iodide is Organically Bound by the Intact Thyroid Gland.* In the experiment recorded in TABLE 1, rats were injected intravenously with a tracer\* dose of radio-iodide. The thyroids were removed at intervals of 15 minutes to 50 hours after the injection and homogenized with cold 10 per cent trichloroacetic acid. The homogenate was centrifuged and washed once with trichloroacetic

\* The term "tracer dose of radioactive iodine" as used here refers to a sample of  $I^{131}$  containing negligible amounts of the naturally-occurring stable iodine isotope  $I^{127}$ .

acid. The residue was hydrolyzed and then separated into thyroxine and diiodotyrosine fractions by a method previously described.<sup>5</sup>

As early as 15 minutes after the injection, 95 per cent of the radioactivity present in the thyroid was precipitable with trichloroacetic acid and therefore presumed to be organically bound. At this early interval, the greatest part of the activity, about 80 per cent, was already in the diiodotyrosine fraction, whereas 10–15 per cent was in the thyroxine fraction.

*How the Gland's Iodine is Distributed between Thyroxine and Diiodotyrosine.* It is well known that diiodotyrosine constitutes the largest fraction of the gland's total iodine. The exact values ascribed to the diiodotyrosine content depend upon the procedure used for the separation of thyroxine from diiodotyrosine. The acid-separation procedure of Harington and Randall<sup>6</sup> yields lower values for the diiodotyrosine content of the gland than does the butyl-alcohol fractionation procedure of Lealand and Foster<sup>7</sup> or of Blau.<sup>8</sup>

The data in TABLE 1 were obtained with the aid of a butyl-alcohol separation procedure adapted in this laboratory to small quantities of thyroid tissue such as that present in a single rat.<sup>5</sup> The distribution of the gland's  $I^{127}$  between thyroxine and diiodotyrosine was found to be strikingly constant. The percentage of the gland's iodine present in the thyroxine fraction varied from 23 to 29; the mean was 26, and its standard error 0.4. This finding agrees well with previous reports from our laboratory in which it was demonstrated that not only is there a constant percentage of iodine present in the gland as thyroxine, but this percentage remains constant even when the total gland iodine is augmented by increasing the iodine intake.<sup>9</sup>

In these same rats, there was also a fairly constant distribution of *radio-iodine* between the thyroxine and diiodotyrosine fractions of the thyroid at each interval. This is not surprising in view of the constancy in the distribution of naturally-occurring  $I^{127}$  between these two compounds.

*Specific Activity-Time Relations as Evidence of the Metabolic Interrelations between Thyroxine and Diiodotyrosine.* Harington<sup>10</sup> has recently reviewed the evidence in support of the view that diiodotyrosine is the biological precursor of thyroxine in the thyroid gland. An important link in this chain of evidence is provided by studies in which the specific activities\* of thyroxine iodine and diiodotyrosine iodine were compared at different intervals after the injection of radio-iodine. Such an experiment was first carried out by Mann, Leblond, and Warren,<sup>11</sup> who injected 6 dogs intravenously with a tracer dose of radioactive iodine and sacrificed them in groups of 2 at the following intervals: 0.5, 8, and 48 hours. The uptake of radio-iodine by the thyroids of these dogs varied

\* Specific activity in this case denotes the number of radioactive units of iodine found in a given fraction per unit weight of non-radioactive (i.e., total) iodine in that fraction.

TABLE 1  
THE I\* AND I127 CONTENT OF THYROXINE AND DIODOTYROSINE FRACTIONS OF THE RAT THYROID AT VARIOUS INTERVALS  
AFTER THE INTRAVENOUS INJECTION OF A TRACER DOSE OF RADIO-IOIDIDE

Interval after injection of I*	Per cent of administered I* recovered in thyroids			Per cent of Total thyroid I* found in		Thyroid I127			
	Total iodine	Thyroxine fraction	Diodo- tyrosine fraction	Thyroxine fraction	Diodo- tyrosine fraction	Thyroxine fraction		Diodotyrosine fraction	
						Amount μg.	Per cent of gland's total I127	Amount μg.	Per cent of gland's total I127
15 min.†	1.6	0.20	1.3	12.5	82.7	3.4	27	8.7	69
1 hr.†	3.9	0.68	3.1	17.5	79.8	3.1	26	8.5	70
4 hrs.†	8.4	1.6	6.6	19.1	79.0	2.9	26	7.6	70
14 hrs.†	19.1	4.9	13.9	25.1	73.1	4.3	28	10.4	67
50 hrs.†	12.2	3.3	8.7	27.0	71.4	2.7	25	7.8	71

† Each line of figures is the average of closely agreeing analyses carried out separately on 3 or 4 rats.



so greatly that it was difficult to compare directly the specific activities of the various fractions in the different dogs. In order to overcome these variations, the specific activity of each iodine fraction of a given dog was calculated as a percentage of the specific activity of the total iodine. The latter values, designated "relative specific activities," were then plotted against time.

There are several aspects of the experiments of Mann *et al.* that are open to criticism. These have been discussed in some detail elsewhere.<sup>12</sup> It need only be pointed out here that most of their difficulties arose from the use of a variable experimental animal, the dog.

The use of a more uniform group of animals such as rats (TABLE 1) obviated most of these difficulties. Since the specific activities of a given iodine fraction agreed fairly well for the rats in each group, it became feasible to plot the averages against time, as shown in FIGURE 1. This en-

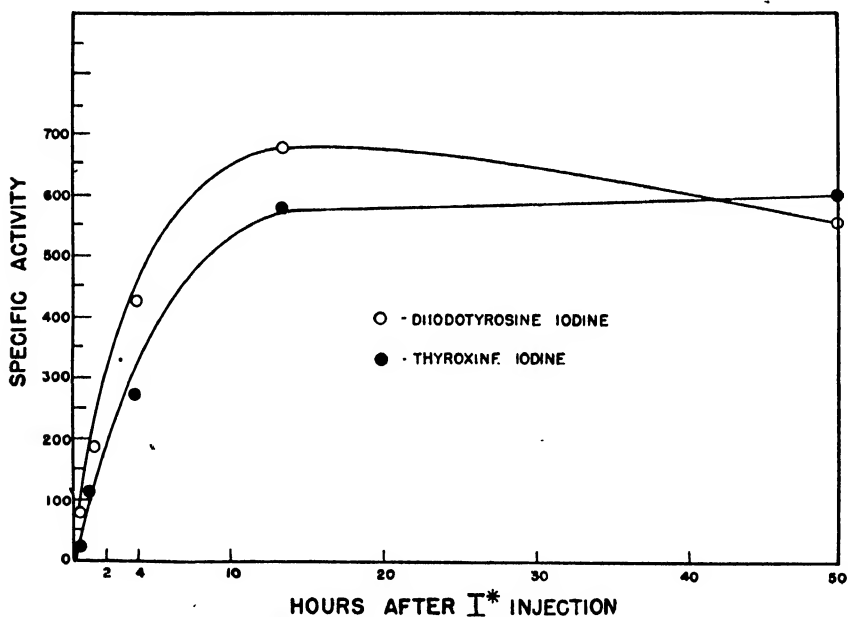


FIGURE 1. Specific activity-time relations of thyroxine iodine and diiodotyrosine iodine in the thyroid glands of rats injected with a tracer dose of  $I^*$ .

abled us to apply the method devised by Zilversmit *et al.*<sup>13</sup> for determining whether one compound in a tissue is a precursor of another. FIGURE 1 shows that the specific activity-time curves of thyroxine iodine and diiodotyrosine iodine follow the general pattern of a precursor and its product as derived by these workers. Thus, the specific activity of the diiodotyrosine iodine remained higher than that of the thyroxine iodine until the maximum of the thyroxine specific activity was reached, at which time the specific activity of the diiodotyrosine iodine fell below

that of the thyroxine iodine. This may be taken as strong evidence that diiodotyrosine is the natural precursor of thyroxine.

It was shown by Zilversmit *et al.*<sup>13</sup> that curves of the type shown in FIGURE 1 can also be used to calculate the time required for the complete renewal of thyroxine in the gland (turnover time). When this calculation was carried out with the data recorded in FIGURE 1, the turnover time of thyroxine amounted to approximately 24 hours. This means that the thyroid gland secreted every 24 hours an amount of thyroxine equal that present in the gland. Since the average thyroxine iodine content of the thyroid gland was 3.3 micrograms, the rate of thyroxine iodine secreted, as calculated by this method, is approximately 1.5 micrograms per 100 gm. of body weight per 24 hours. This value is lower than that reported by Dempsey and Astwood (5.2 micrograms *l*-thyroxine per 100 gm. body weight per day<sup>14</sup>), but higher than that estimated by Griesbach and Purves (2.25 micrograms *dl*-thyroxine per 100 gm. body weight per day<sup>15</sup>). These previous estimates are based on methods which depend on the replacement of circulating thyroid hormone by injections of crystalline thyroxine.

### Studies with Surviving Slices of Thyroid Tissue

The demonstration that iodine concentration and synthesis of thyroxine and diiodotyrosine can be made to take place in *surviving slices* of thyroid tissue is a most interesting outcome of the biological application of the radioactive isotope of iodine.<sup>16</sup> This was made possible by the sensitivity of the radioactive procedure, which is such as to permit the accurate measurement of traces of newly synthesized thyroxine and diiodotyrosine. The results provided by the use of this new tool have served to shed new light on the mechanism of the biosynthesis of thyroxine and diiodotyrosine.

*Iodine-Concentrating Capacity of Thyroid Tissue Slices.* A comparison of the iodine-concentrating capacity of surviving slices of thyroid gland, liver, and spleen is presented in TABLE 2. 300 mg. of each tissue was sus-

TABLE 2  
IODINE CONCENTRATION BY SURVIVING LIVER, SPLEEN, AND THYROID SLICES

Tissue	Iodide <sup>127</sup> I added to Ringer's, $\mu$ g.	Time of incubation, hours	Per cent of Ringer's <sup>127</sup> I* recovered in slices	Micrograms of Ringer's <sup>127</sup> I incorporated into slices
Sheep liver	0†	1	2.4	
Sheep thyroid	0†	1	81.4	<0.3
Beef spleen	0†	2	1.5	
Beef thyroid	0†	2	87.9	<0.3
Sheep thyroid	0†	1	86.2	<0.3
Sheep thyroid	20	1	61.3	12

† When no iodide was added, the Ringer's medium contained less than 0.3 microgram of iodine.

pended in 3 cc. of a Ringer-bicarbonate solution containing a tracer amount of radioactive iodide. That excised thyroid tissue can retain its extraordinary capacity for accumulating iodine is well brought out by the finding that as much as 80 per cent of the  $I^*$  that had been added to the medium was removed by the 300 mg. of thyroid slices in one hour. In 2 hours, close to 90 per cent of the  $I^*$  was found within the slice. In similar intervals, less than 5 per cent of the medium's  $I^*$  was taken up by 300 mg. of liver or spleen.

The most rapid uptake of the added  $I^*$  by the surviving thyroid slices occurred during the first 10 minutes (approximately 40 per cent). By the end of 30 minutes, approximately 60 per cent of the  $I^*$  of the Ringer's medium had entered the slices.

In the experiments described above, no  $I^{127}$  was added to the Ringer's medium. The traces of  $I^{127}$  present in the reaction flask were due to the impurities of the reagents used in the preparation of the Ringer's solution. The addition, however, of a known quantity of iodide  $I^{127}$  to the Ringer's solution in which the thyroid slices are suspended, permitted the measurement of the absolute amounts of  $I^{127}$  that entered the thyroid slice. The results are shown in TABLE 2. Thus, approximately 12 micrograms of iodide entered the slices in one hour when 20 micrograms were added to the Ringer's medium.

*The IN VITRO Synthesis of Thyroxine and Diiodotyrosine.* When 225 mg. of surviving sheep thyroid slices were incubated for 2 hours in a Ringer's medium to which a tracer dose of radioactive iodide had been added, it was found that as much as 85 per cent of the labeled iodine was organically bound, about 75 per cent as diiodotyrosine and about 10 per cent as thyroxine (TABLE 3). The thyroid iodine was fractionated by a

TABLE 3  
THYROXINE AND DIIODOTYROSINE FORMATION BY SHEEP THYROID TISSUE  
AND BY DESICCATED THYROID POWDER

Form of thyroid tissue	Weight of preparation used, mg.	Volume of Ringer's solution, cc.	Incubation period, hours	Per cent of Ringer's $I^*$ recovered as	
				Thyroxine	Diiodotyrosine
Slices	225	4	2	9.6	76.8
Homogenate†	225	4	2	1.5	8.7
Desiccated powder	50	4	2	0.7	7.0

† Prepared in all-glass homogenizer.

modification of Blau's butyl-alcohol procedure.<sup>8</sup>

*Cytochrome-Cytochrome Oxidase System Participates in the Synthesis of Thyroxine and Diiodotyrosine by Thyroid Tissue.* Tissue organization (TABLE 3) was found to be of utmost importance in the *in vitro* conversion of inorganic iodide to thyroxine and diiodotyrosine.<sup>16</sup> Little formation of either compound was observed when tissue organization was disrupted

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by homogenization, a finding that suggests the participation of an intracellular enzyme system in these syntheses.

Evidence that oxidative enzymes are involved was obtained by a study of the effects of anaerobiosis and of substances that are inhibitors of cytochrome oxidase.<sup>17</sup> The results are shown in TABLE 4. Sulfide, azide, and

TABLE 4  
EFFECT OF VARIOUS INHIBITORS ON SYNTHESIS OF THYROXINE AND DIIODOTYROSINE  
BY SURVIVING THYROID TISSUE SLICES AS MEASURED WITH I\*

Inhibitor	Concentration in Ringer's solution or in atmosphere above solution	Per cent inhibition	
		Thyroxine formation	Diiodotyrosine formation
RESPIRATORY INHIBITORS:			
Azide	0.005M	90	89
Cyanide	0.01M	94	83
Sulfide	0.003M	89	85
Anaerobiosis	95 per cent N <sub>2</sub> 5 per cent CO <sub>2</sub>	67	54
Carbon monoxide	90 per cent CO 5 per cent CO <sub>2</sub> 5 per cent O <sub>2</sub>	71 (in dark) 46 (in light)	81 (in dark) 39 (in light)
GOITROGENIC SUBSTANCES:			
Thiouracil	0.001M	95	90
Sulfanilamide	0.001M	47	58
<i>p</i> -Aminobenzoic acid	0.001M	73	79

cyanide in low concentrations had strong inhibitory effects on the formation of thyroxine and diiodotyrosine. Only 5–15 per cent of the normal synthesis occurred in the presence of these agents. The complete absence of oxygen or the presence of a high concentration of carbon monoxide in the atmosphere above the Ringer's solution also had marked inhibitory effects on the organification of the Ringer's I\*. The inhibitory effect of the carbon monoxide was partially reversible in the presence of strong light. The inhibition of the incorporation of I\* into thyroxine and diiodotyrosine by each one of these poisons, together with the marked effect of light on the inhibition by CO (which is perhaps the best single piece of evidence for this point) strongly suggests that the cytochrome-cytochrome oxidase system is involved in the formation of these iodine compounds by the thyroid gland.

*The Effect of Goitrogenic Substances on the IN VITRO Synthesis of Thyroxine and Diiodotyrosine.* The *in vitro* technique for measuring the conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine provided a method for testing directly the action of the goitrogenic compounds on the thyroid glands.<sup>18</sup> As shown in TABLE 4, when surviving thyroid slices were incubated in the presence of very low concentrations of thiouracil, sulfanilamide, and *p*-aminobenzoic acid, there was a marked depression in the formation of both diiodotyrosine and thyroxine. Many

other antithyroid compounds have similar inhibitory effects on this *in vitro* reaction.<sup>19</sup> These findings are in accord with the observations of MacKenzie and MacKenzie,<sup>20</sup> as well as of Astwood *et al.*,<sup>21</sup> who concluded from experiments on intact animals that such antithyroid compounds as thiouracil and sulfanilamide exert their effects by interfering with the synthesis of thyroid hormone.

*The Question of Exchange.* In the foregoing sections, it was assumed that the appearance of radioactive iodine in the organic iodine fractions of the thyroid indicates the formation of new molecules of diiodotyrosine and thyroxine. The possibility, however, that simple exchange reactions occur must be considered. Exchange, in the present instance, may be defined as the formation of radiothyroxine or radiodiiodotyrosine from radioactive inorganic iodide and pre-formed non-radioactive thyroxine or diiodotyrosine molecules. The conditions necessary to make exchange occur between molecular iodine and diiodotyrosine and between iodide ions and diiodotyrosine have been recently determined by Miller *et al.*<sup>22</sup> Convincing evidence, however, that the incorporation of inorganic radio-iodide into thyroxine and diiodotyrosine as measured here is *not* simply an exchange reaction, but represents the formation of new molecules of these substances, is provided by the following experimental findings:

(1) When surviving thyroid slices were incubated in a Ringer medium to which radioactive iodide had been added, a large part of the radio-iodine was soon incorporated into thyroxine and diiodotyrosine. Organic binding of iodine was greatly reduced, however, when tissue organization was disrupted by homogenization; in this case, nearly all of the radio-iodine was recovered in the inorganic iodine fraction (TABLE 3).

(2) The conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine by surviving thyroid slices was greatly inhibited under anaerobic conditions or in the presence of cytochrome oxidase inhibitors. These findings strongly imply the participation of an intracellular enzyme system in the formation of these iodinated compounds.

(3) It has been shown by the MacKenzies and by Astwood *et al.* that goitrogenic compounds depress hormone formation by the thyroid gland. These same compounds were found to inhibit both the *in vitro* and the *in vivo* conversion of inorganic radioactive iodide to thyroxine and diiodotyrosine by thyroid tissue. A single injection of 10 mg. of thiouracil into a rat completely abolished, for some time, the thyroid's capacity of converting circulating radioactive iodide to diiodotyrosine and thyroxine.

### *The Mechanism of Iodine Fixation by the Thyroid Gland*

It was pointed out in the first section of the paper that, as early as 15 minutes after the injection of a *tracer dose* of radio-iodide into rats, over 90 per cent of the radioactivity in the thyroid gland is organically bound. It would appear from this observation that the selective uptake of iodine

by the thyroid depends on the ability of this tissue to convert iodine to diiodotyrosine and thyroxine. This mechanism, however, will not account for iodine concentration by the thyroid when relatively *large amounts* of iodine, rather than tracer doses, are injected. Thus, Leblond observed that, after the injection into guinea pigs and rats of 500 micrograms of labeled iodide per 100 gm. body weight, the largest part of the iodine fixed by the thyroid remained as inorganic iodide for some time.<sup>23</sup> Lein<sup>24</sup> reported that, after the intravenous injection of 35 micrograms of labeled iodine into a rabbit, most of the iodine fixed by the thyroid during the first 10 minutes appeared in the inorganic fraction (acetone-soluble). These findings have been confirmed in our laboratory. One hundred micrograms of labeled iodine was injected into rats and their glands removed one hour later; at this interval, approximately 50 per cent of the iodine taken up by the gland was in the inorganic fraction (trichloroacetic acid-soluble). These findings make it appear that there is some mechanism in the thyroid gland for concentrating inorganic iodine which is not dependent upon its conversion to organic iodine.

Further evidence was provided by the use of antithyroid substances. With the aid of these compounds, it was possible to separate almost completely the iodine-concentrating capacity of thyroid tissue from the mechanism for iodine organification. This was first shown in a study in which surviving thyroid slices were suspended in an oxygenated Ringer medium containing a tracer dose of  $I^*$ . Although the capacity of the slices to convert the  $I^*$  to organically-bound iodine was greatly depressed in the presence of 0.001M thiouracil, the latter had only slight effect on the uptake of  $I^*$  by the thyroid slices.<sup>18</sup>

Subsequently, several investigators have demonstrated that the enlarged thyroid gland of the thiouracil-treated rat, though lacking the capacity to synthesize organic iodine compounds, retains its capacity to concentrate iodine.<sup>25, 26</sup> The iodine taken up under these conditions is not bound to protein and, as shown by Vanderlaan and Bissell,<sup>27</sup> does not remain in the gland very long. Their curves depicting the uptake of iodine by the thyroids of propylthiouracil-treated rats at various times after the injection of 50 or 500 micrograms of KI are similar in shape to that found for the uptake of iodine by plasma. It seems, therefore, that the iodine concentration observed in the goitrogen-inhibited thyroid is proportional to the iodine level in the circulating fluid. However, since the level of iodine in the gland rises to several hundred times that of the plasma, there can be little doubt that some type of selective mechanism for concentrating iodine is still operating in this inhibited gland.

It is shown in FIGURE 2 that the iodine-concentrating mechanism of the propylthiouracil-inhibited thyroid does not depend upon the injection of a large dose of iodine. Its operation is also demonstrable when a *tracer dose* of labeled iodide is injected. Under these circumstances, almost 9 per cent of the injected  $I^*$  was found in the thyroid glands in one hour, whereas in 24 hours only two per cent was found there. At both

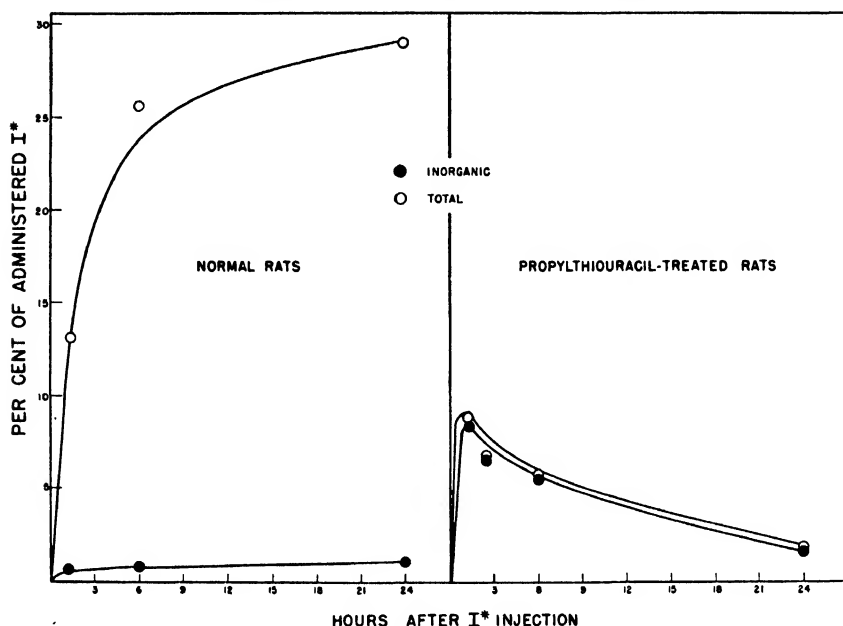


FIGURE 2. The uptake of a tracer dose of  $I^*$  by the thyroids of normal and propylthiouracil-treated rats.

time intervals, practically all of the radioactive iodine was recovered in the inorganic fraction. In control rats, only about 4 per cent of the radioiodine in the thyroid was in the inorganic fraction at the one-hour interval.

When 100 micrograms of labeled iodine was injected into propylthiouracil-treated rats (FIGURE 3), over 10 per cent of it appeared in the thyroids in half an hour. Thereafter, the iodine concentration fell off rapidly, and in 26 hours had diminished almost to its original value. All of the radioiodine in the gland was in the form of inorganic iodide. This was shown by the finding that it was readily oxidized to  $I_2$  by excess iodate in a slightly acidic solution. None of it precipitated with the proteins upon treatment of the thyroid tissue with trichloroacetic acid, and all of it passed readily through a dialysis membrane.

From the above experiments, it appears that iodine can be initially fixed in the thyroid gland by some mechanism that is distinct from the reactions responsible for converting it to an organic form.

### *Circulating Iodine*

#### *Plasma Protein-Bound Iodine; Its Metabolic Significance*

Although the exact nature of the circulating thyroid hormone remains to be determined, the concentration of the fraction of plasma iodine designated *protein-bound* has gained wide use as an indicator of thyroid activity in man.<sup>28-32</sup> This fraction of plasma iodine is the iodine

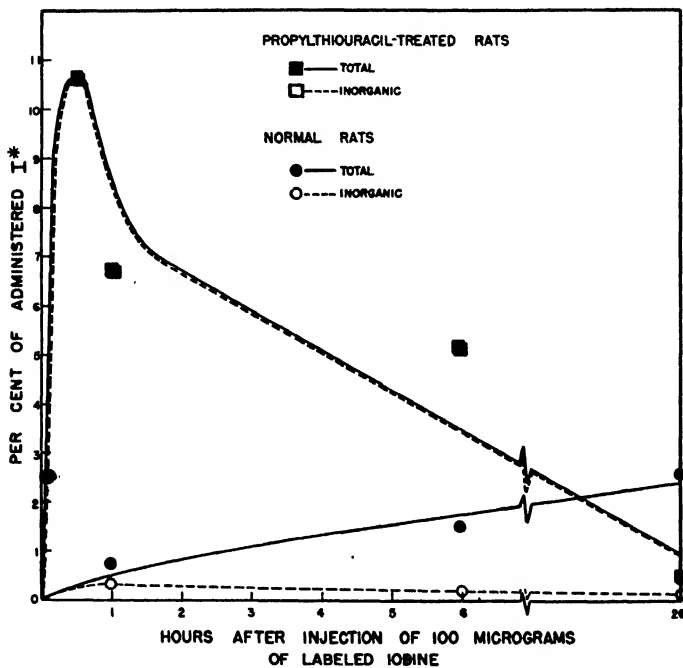


FIGURE 3. The uptake of 100 micrograms of labeled iodine by the thyroids of normal and propylthiouracil-treated rats.

which is precipitated from plasma along with its proteins by such agents as tungstic acid, zinc hydroxide, or acetic acid in the presence of heat and which, in addition, cannot be freed from these proteins by simple washing. Investigations with radioactive iodine have helped to throw light on the metabolic significance of this iodine fraction of plasma.<sup>33</sup> Such studies are described in this section.

*Rate of Formation of Plasma Protein-Bound Iodine in Thyroidectomized Rats as Measured with Radioactive Iodine.* The rate at which injected radioactive inorganic iodide is converted to protein-bound iodine of plasma is shown in TABLE 5. After its introduction into *normal* rats maintained on a low iodine intake, radioactive inorganic iodide makes its appearance rapidly in the protein-bound iodine fraction of plasma. In 13 hours, 50 per cent of the plasma I\* was found to be protein-bound, in 25 hours 90 per cent.

In the *completely thyroidectomized* rat, on the other hand, only 5 to 10 per cent of the plasma I\* was protein-bound as late as 25 hours after the injection (TABLE 5). These results indicate that the formation of protein-bound iodine is greatly diminished in completely thyroidectomized rats.

As was to be expected, thyroidectomy also depressed the concentration of protein-bound iodine in plasma (TABLE 6). The level of this con-



TABLE 5  
FORMATION OF PLASMA PROTEIN-BOUND I\* BY THYROIDECTOMIZED RAT†

Expt.	Treatment	Interval when animals sacrificed		Plasma		
		After operative procedure, hours	After I* injection, hours	Total I* as per cent injected dose per 10 cc. plasma	Protein-bound I* as per cent of injected dose per 10 cc. plasma	$\frac{\text{Protein-bound I}^*}{\text{Total I}^*} \times 100$
1	Sham Operated Control	60-72	13	1.1	0.54	49
	Sham Operated Control	60-72	13	0.96	0.65	68
	Thyroidectomized	60-72	13	3.0	0.16	5.3
	Thyroidectomized	60-72	13	2.9	0.17	5.9
	Thyroidectomized	60-72	13	2.8	0.22	7.9
2	Sham Operated Control	64-72	25	1.1	0.94	85
	Sham Operated Control	64-72	25	1.3	1.1	88
	Sham Operated Control	64-72	25	1.2	1.1	94
	Sham Operated Control	64-72	25	2.0	1.8	91
	Thyroidectomized	64-72	25	2.4	0.18	7.5
	Thyroidectomized	64-72	25	2.0	0.15	7.5
	Thyroidectomized	64-72	25	1.4	0.083	5.9

† The completeness of all thyroidectomies was checked by the radioautograph procedure.

TABLE 6

PLASMA PROTEIN-BOUND IODINE OF RATS 3 AND 16 DAYS AFTER THYROIDECTOMY

<i>Controls, micrograms per cent</i>	<i>3 days after thyroidectomy, micrograms per cent</i>	<i>16 days after thyroidectomy, micrograms per cent</i>
3.1	1.2	1.3
3.1	0.9	1.2
3.6	1.1	1.1

stituent fell in 3 days after thyroidectomy from 3.3 micrograms per cent (average control value) to values of about 1.0 microgram per cent. The significance of these low values, however, is difficult to assess, since they depend upon colorimetric readings which differ only slightly from those given by the reagent blanks. Whether there exists, in the completely thyroidectomized rat, a small amount of protein-bound iodine must therefore remain an open question.

*Rate of Formation of Plasma Protein-Bound Iodine in Animals Injected with Thyrotropic Hormone.* The rate of appearance of injected radioactive inorganic iodide in protein-bound iodine of plasma of hyperthyroid animals is shown in TABLE 7. A striking increase above control values was observed as early as 3 hours after I\* injection. At this interval, the percentage of the injected I\* found in the protein-bound iodine fraction per 10 cc. of plasma varied from 0.73 to 2.9 in hyperthyroid rats and from 0.17 to 0.29 in the control rats. In the former, 39–73 per cent of the plasma I\* was protein-bound, whereas in the controls only 4.1–9.2 per cent was so bound. Large differences between the two groups were also evident 6 hours after the injection of the radioactive isotope; at this interval, 11–32 per cent of the plasma I\* was protein-bound in the control rats, as compared with 82–87 per cent in the hyperthyroid rats. A similar increase was also observed in hyperthyroid guinea pigs.

The effect of injection of thyrotropic hormone on the level of protein-bound iodine of plasma is shown in TABLE 8. Significant increases were observed both in the rats and in the guinea pigs. In the latter, the protein-bound iodine level rose from a mean value of 2.1 micrograms per cent to one of 5.8 micrograms per cent, an increase of 175 per cent.

In summary, it may be stated that, with respect to both its concentration and—what is new—its *rate of formation*, protein-bound iodine of plasma responds to experimentally induced changes in thyroid activity in a manner to be expected in the case of a circulating thyroid hormone. Thus, not only was the level of protein-bound iodine of plasma elevated by the injection of thyrotropic hormone and depressed by the removal of the thyroids, but the rate of appearance of injected radioactive inorganic iodide in the protein fraction of plasma was greatly diminished by thyroidectomy and greatly augmented by injection of thyrotropic hormone. *This rate of appearance may prove as useful as the level of protein-bound iodine for testing thyroid activity.*

TABLE 7

FORMATION OF PLASMA PROTEIN-BOUND I\* BY RATS AND GUINEA PIGS INJECTED WITH THYROTROPIC HORMONE

Animal	Interval after I* injection, hours	Controls			Thyrotropic hormone†		
		Weight of thyroid glands, mg.	Protein-bound I* as per cent of injected dose per 10 cc. plasma	$\frac{\text{Protein-bound I*}}{\text{Total I*}} \times 100$	Weight of thyroid glands, mg.	Protein-bound I* as per cent of injected dose per 10 cc. plasma	$\frac{\text{Protein-bound I*}}{\text{Total I*}} \times 10$
Rats	3	23	0.29	7.3	36	2.9	73
	3	22.5	0.21	5.2	30	1.3	39
	3	29	0.17	4.1	76	0.73	53
	3	33.5	0.26	9.2			
	6	31.5	0.44	30	39	2.0	82
	6	19	0.36	11	49	4.1	88
	6	33	0.34	27	43.5	3.1	87
	6	38	0.31	32			
Guinea pigs	16	75	0.0041	7.5	113	0.070	77
	16	56	0.0045	12	147	0.14	83
	16	68	0.0031	12	106	0.11	77
	16	68	0.0064	18	120	0.13	81
					112	0.099	86

† The rats were injected with 10 mg. of thyrotropic hormone twice daily for 4 days. The 3-hour and 6-hour groups also received one injection on the 5th day at the same time that the I\* was injected. The guinea pigs were treated as described in TABLE 8.

TABLE 8  
EFFECT OF THYROTROPIC HORMONE ON CONCENTRATION  
OF PROTEIN-BOUND IODINE IN PLASMA

Animal	Treatment	Weight of thyroid glands, mg.	Plasma Protein-bound iodine, micrograms per cent
Rats	Controls	33	3.2
	Controls	28	3.2
Rats	{ 3 injections with thyrotropic hormone over 24-hour period, each injection 10 mg.†	34	4.9
		45	6.2
		43	5.3
		43	5.7
Guinea pigs	Controls	75	2.2
	Controls	56	2.0
Guinea pigs	{ 2 mg. thyrotropic hormone in- jected once daily for 3 days and twice on 4th day. Animals sacrificed morning of 5th day.	113	6.1
		147	6.1
		106	4.4
		120	6.8

† The thyrotropic hormone preparation contained 4 guinea pig units per mg.

### The Thyroxine-like Fraction of Plasma

The small amount of iodine in plasma makes difficult its exact chemical identification. Although values for the thyroxine content of blood have been reported,<sup>34</sup> their accuracy must remain doubtful until confirmation is provided by other investigators.

In contrast to the chemical measurement of thyroxine, determinations of *radiothyroxine* need not suffer any loss in accuracy when this iodine fraction is present in very minute quantities. The accuracy of the separation, when only radioactive measurements are involved, can be maintained by the addition of non-radioactive carriers. Thus, in the solution of certain problems, the judicious use of the radioactive isotope of iodine has circumvented the technical difficulties inherent in the chemical measurement of very minute concentrations of iodine.

In a study carried out in this laboratory by Morton *et al.*, plasma was removed from normal rats at intervals after the injection of radioiodide.<sup>35</sup> The plasma was hydrolyzed with 2N NaOH and, after the addition of non-radioactive thyroxine, diiodotyrosine, and inorganic iodide as carriers, was subjected to a butyl-alcohol fractionation. The radioactivity was separated into the following 3 fractions by the procedure previously outlined: thyroxine-like, diiodotyrosine-like, and inorganic.

The curves in FIGURE 4 show the distribution of the plasma radioactive iodine among these 3 fractions. A significant fact emerging from this experiment is that, as early as 24 hours after the injection, 80 per cent of the radioactive iodine of plasma is contained in the thyroxine-like fraction.

For a different group of rats, the rate of appearance of the injected

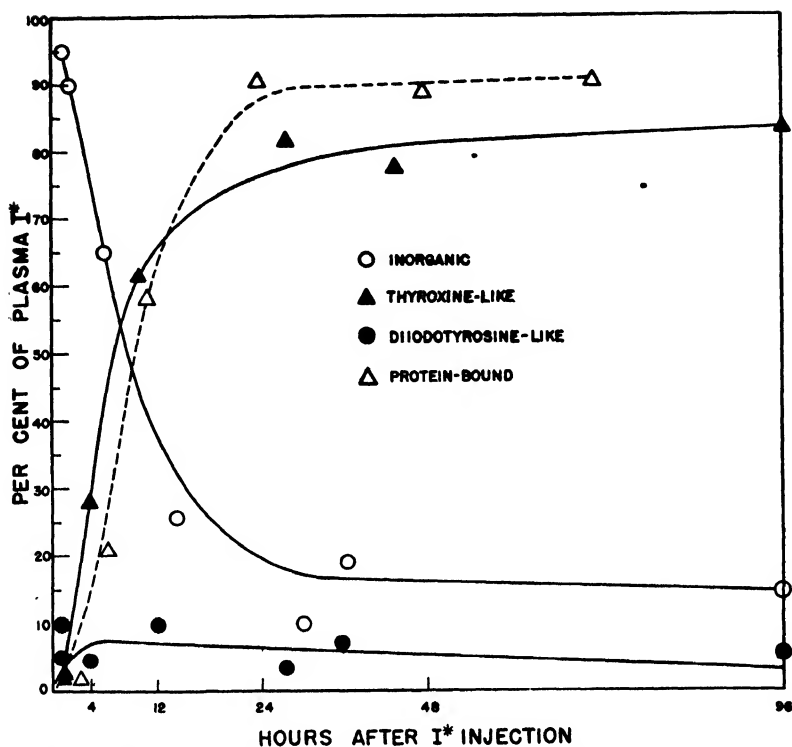


FIGURE 4. The distribution of plasma I\* among the various iodine fractions. For explanation, see text.

radio-iodide in the protein-bound iodine fraction of plasma has also been plotted on the same axes (FIGURE 4). The resemblance between the curves for protein-bound iodine and thyroxine helps to throw light on the nature of hormonal iodine. The similarity in the rates of incorporation of radio-iodine in these two fractions suggests that protein-bound iodine of plasma is largely composed of some thyroxine-like iodine compound attached to protein. The finding that much or all of the protein-bound iodine of plasma is extractable with organic solvents also supports the view that the circulating thyroid hormone is in the form of a small thyroxine-like molecule loosely attached to protein.<sup>36, 37</sup>

It is interesting to note that, in the experiments described above, only 5–10 per cent of the plasma radioactivity was found in the diiodotyrosine fraction at all intervals up to 96 hours after the injection. Since, in the thyroid gland, the amount of newly formed radiiodiodotyrosine exceeds that of newly formed radiothyroxine, this finding demonstrates a preferential output of thyroxine into plasma by the gland.

### *The Role of the Anterior Pituitary in Iodine Metabolism*

Much has been gleaned from morphological studies on the nature of

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the hypophyseal control of thyroid function, but an understanding of the relation of thyrotropic hormone to iodine metabolism and thyroxine synthesis by the thyroid gland has had to await the introduction of radioactive iodine as a labeling agent and the development of micro-methods for the measurement of iodine and thyroxine. The recent work made possible by these new tools is reviewed below.

### *The Hypophysectomized Animal*

*The Iodine Content of Gland and Plasma.* The effects of hypophysectomy upon the concentration of iodine in plasma and thyroid gland of the rat are shown in TABLE 9. A decrease in the level of plasma iodine was

TABLE 9  
EFFECT OF HYPOPHYSECTOMY ON THYROID AND PLASMA IODINE

Days after hypophysectomy	Thyroid weight per 100 gm. body weight, mg.	Thyroid iodine				Plasma iodine, µg. per cent	
		Thyroxine		Total		Protein-bound	Total
		Amount, µg.	Concentration, mg. per cent	Amount, µg.	Concentration, mg. per cent		
Control	2	13.0	2.3	6.8	19	2.7	
Operated		10.0	2.2	6.8	26	2.3	
Control	4	13.5	2.7	8.5	26	2.6	2.5
Operated		11.1	3.5	11.9	45	1.2	1.2
Control	9	10.9	1.9	6.9	26	2.6	2.7
Operated		7.5	2.2	8.6	51	1.1	1.5
Control	44	10.1	2.8	9.0	38	3.5	
Operated		6.7	3.1	10.2	76	1.9	
Operated	1 yr.	8.6	2.3	15	63	1.9	

observed as early as 2 days after hypophysectomy, and by 4 days a decrease of about 50 per cent had occurred in both total and protein-bound iodine of plasma. No further significant drop was observed in rats examined 44 days after hypophysectomy.

A decrease in the weight of the thyroid gland was detected as early as 2 days after hypophysectomy and was quite pronounced in 9 days. However, at no time up to one year after hypophysectomy was a decrease observed in the amount of thyroxine or of total iodine contained in the gland (TABLE 9). This resulted in a greatly increased concentration of iodine in the gland of the hypophysectomized rat; thus, in 44 days, the thyroid glands of the operated and control rats contained, respectively, 0.076 and 0.038 per cent total iodine and 0.023 and 0.012 per cent thyroxine iodine.

The observation that the level of protein-bound iodine in plasma falls as early as 48 hours after excision of the pituitary gland is not surprising in view of the well-known effects of hypophysectomy upon the morphol-

ogy of the thyroid gland. The presence, however, of normal and even greater than normal amounts of thyroxine in the gland during the time that this is occurring, throws new light on the mechanism by which thyroxine is released into the circulation by the gland. These findings leave no doubt that a lowered thyroxine content of plasma is not a *direct* stimulus to the thyroid gland to release its thyroxine. They demonstrate quite clearly that, even in the gland that contains an amount of thyroxine greater than normal, its release into the bloodstream requires the action of the thyrotropic hormone.

*Studies with the Radioactive Isotope of Iodine.* The data presented above may be considered to provide a picture of the steady state of iodine metabolism in the hypophysectomized rat. The conclusions derived from such data have been supplemented considerably by the dynamic picture obtained with the use of radioactive iodine.<sup>35</sup>

Tracer doses of I\* were injected into hypophysectomized and normal rats and the fate of this iodine compared. The injection of a tracer dose of I\* (*i.e.*, carrier-free) does not increase the iodine content of the animal. At an early hour, a much smaller amount of the *initially labeled iodide* appeared in the thyroid gland of the hypophysectomized than in that of the normal rat (FIGURE 5). Thus, in 4 hours, the normal thyroids contained approximately 60 per cent of the injected I\*, while at the same time-interval the gland of the hypophysectomized rat contained less than 5 per cent. Moreover, the character of the curves depicting the uptake of the labeled iodine differed in the two types of rats. The gland of the hypophysectomized rat should not be regarded as completely inactive, however, for it showed a continuous rise in its content of the injected I\* during the entire period of observation and at 96 hours held about 25 per cent of the administered I\*.

A striking difference between normal and hypophysectomized rats was also observed in the manner in which injected I\* disappeared from the plasma (FIGURE 5). The I\* left the blood more rapidly in the former, in keeping with the more rapid uptake of I\* by the thyroids. The rise in the I\* content of the plasma of normal rats after 12 hours (not observed in the hypophysectomized rats) reflects the release of labeled hormone from the thyroids.

In order to determine the fate of the I\* that enters the gland, this iodine was separated into three fractions: diiodotyrosine, thyroxine, and inorganic iodide. The distribution of the I\* of each gland among these three fractions is shown in FIGURE 6. The rapidity with which iodine that enters the *normal* gland is organically bound is again clearly brought out. At the 4-hour interval, about 90 per cent of the I\* contained in the normal gland is organically bound, most of it as diiodotyrosine and about 20 per cent as thyroxine. At later intervals (up to 96 hours), the percentage in the form of thyroxine increased, while that in the form of diiodotyrosine decreased.

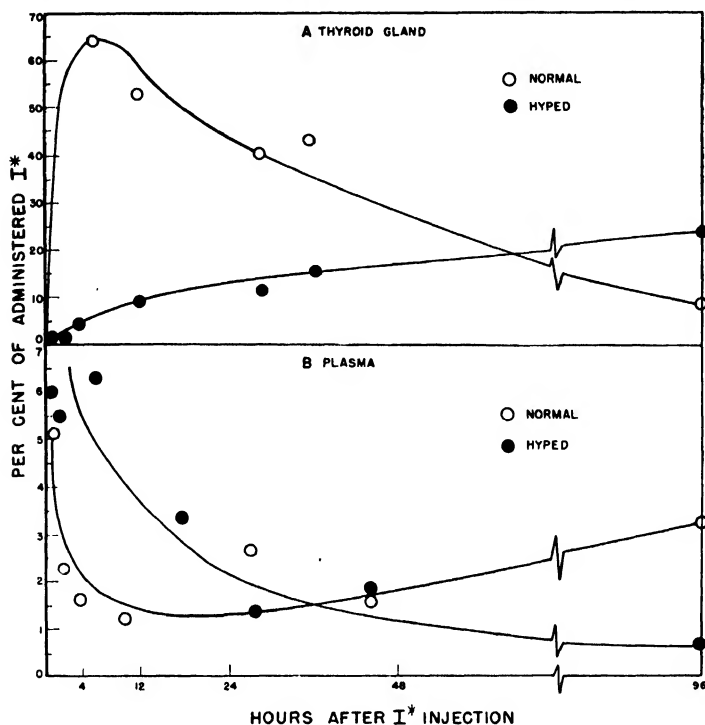


FIGURE 5. The uptake of total  $I^*$  by the whole thyroid gland (A) and by entire plasma (B) of the normal and hypophysectomized rat. Entire plasma was estimated as 5 per cent of the total body weight. A tracer dose of  $I^*$  was injected intraperitoneally into each rat.

FIGURE 6 shows that whatever iodine does enter the thyroid gland of the hypophysectomized animal is rapidly converted to diiodotyrosine. About 80 per cent of the gland's  $I^*$  was present as diiodotyrosine as early as 4 hours, but (interestingly enough) the same percentage of the gland's radioactive iodine was present as diiodotyrosine as late as 96 hours after the injection of the isotopic iodine. The conversion of diiodotyrosine to thyroxine appears to be interfered with in the absence of hypophysis. This interpretation of the data is fully supported by the finding (FIGURE 7) that practically none of the plasma  $I^*$  of the hypophysectomized rat appears as thyroxine iodine even as late as 96 hours, a time when approximately 80 per cent of the  $I^*$  contained in plasma of the normal rat is thyroxine iodine (FIGURE 4).

### *The Animal Treated with Thyrotropic Hormone*

*The Iodine Content of Gland and Plasma.* Many workers have reported a decreased iodine content in the thyroid gland of animals treated with preparations of thyrotropic hormone.<sup>38-43</sup> Closs, Loeb, and MacKay<sup>40</sup> found an increase in blood iodine as well as a decrease in gland iodine in



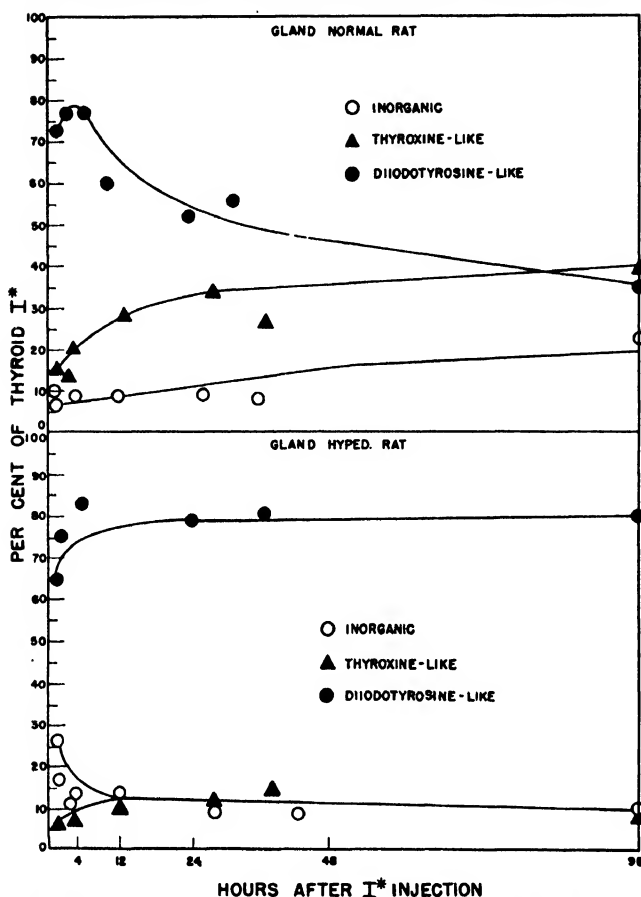


FIGURE 6. The distribution of thyroid  $I^*$  in normal and hypophysectomized rats. Each rat received intraperitoneally a tracer dose of  $I^*$ . For explanation, see text.

guinea pigs injected with a pituitary extract. These findings have been confirmed in this laboratory (TABLE 10, TABLE 8). The data in TABLE 10 show that the decrease in gland iodine results from a fall in the amount of organically-bound iodine (thyroxine and diiodotyrosine) but not of inorganic iodide.

*Studies with Radioactive Iodine—IODINE-CONCENTRATING CAPACITY.* The thyroid glands of animals injected with thyrotropic hormone have a greater than normal capacity for fixing administered  $I^*$ . This has been shown to be the case whether the radioactive iodine was administered as a tracer dose (*i.e.*, carrier-free) or along with carrier. Such increases in uptake have been found in the guinea pig,<sup>44-46</sup> the rabbit,<sup>47</sup> and the rat.<sup>47</sup>

A study by Morton *et al.*<sup>46</sup> revealed that, whereas 4-9 per cent of an injected tracer dose of  $I^*$  was present in the thyroid glands of normal

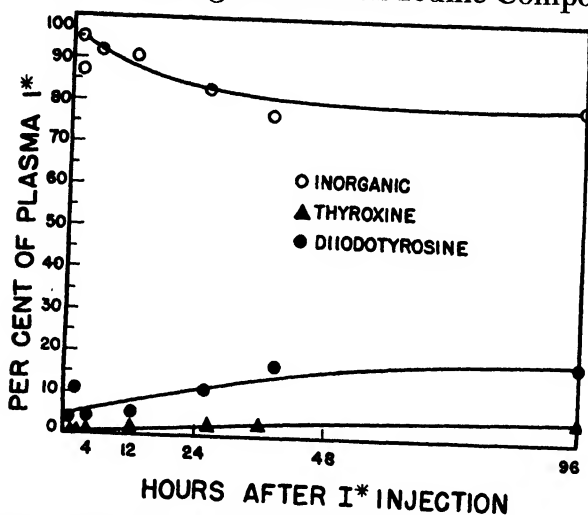


FIGURE 7. The distribution of plasma I\* in the hypophysectomized rat. For explanation, see text.

TABLE 10

THE EFFECT OF THYROTROPIC HORMONE ON THE IODINE ( $I^{127}$ ) FRACTIONS OF THE THYROID GLAND OF THE GUINEA PIG

Weight of gland	Inorganic fraction			Diiodotyrosine fraction			Thyroxine fraction		
	Amount, $\mu$ g.	Concentration, mg. per cent	Per cent of total iodine	Amount, $\mu$ g.	Concentration, mg. per cent	Per cent of total iodine	Amount, $\mu$ g.	Concentration, mg. per cent	Per cent of total iodine
Controls									
75	0.47	0.6	1.3	28.0	37	75	9.0	12	24
56	0.27	0.5	1.1	19.3	34	76	5.8	10	23
68	0.13	0.2	0.4	23.2	34	74	8.2	12	26
68	0.26	0.4	1.2	16.5	24	78	4.3	6.3	20
Hyperthyroid†									
113	1.0	0.9	5.0	14.8	13	74	4.2	3.7	21
147	0.76	0.5	6.3	8.9	6.1	74	2.3	1.6	19
106	0.45	0.4	7.6	4.3	4.1	72	1.2	1.1	20
120	0.80	0.7	6.8	8.6	7.2	73	2.4	2.0	20
112	0.82	0.7	5.2	11.1	10	70	3.9	3.5	25

† Injected with thyrotropic hormone as described in TABLE 8.

guinea pigs 2 hours after the injection, 12-23 per cent was found in the hyperplastic glands of pigs treated with thyrotropic hormone. At the 26-hour interval after the injection of  $I^*$ , 14-27 per cent of the administered labeled iodine was present in the normal thyroids and 32-41 per cent in the hyperactive glands. The increased uptake was apparent in all three iodine fractions of the thyroid gland. Similar results were obtained in more recent experiments shown in FIGURE 8.

THE DISTRIBUTION OF THE  $I^*$  IN THE THYROID GLAND AS THYROXINE AND DIIODOTYROSINE. In the hyperthyroid animal, as in the normal, the  $I^*$ , when administered as a tracer dose, is taken up by the thyroid gland

and rapidly converted there to organic iodine. The distribution of the  $I^*$  within the gland among the 3 fractions, iodide, thyroxine-like, and diiodotyrosine-like, is shown graphically in FIGURE 8. Under the influ-

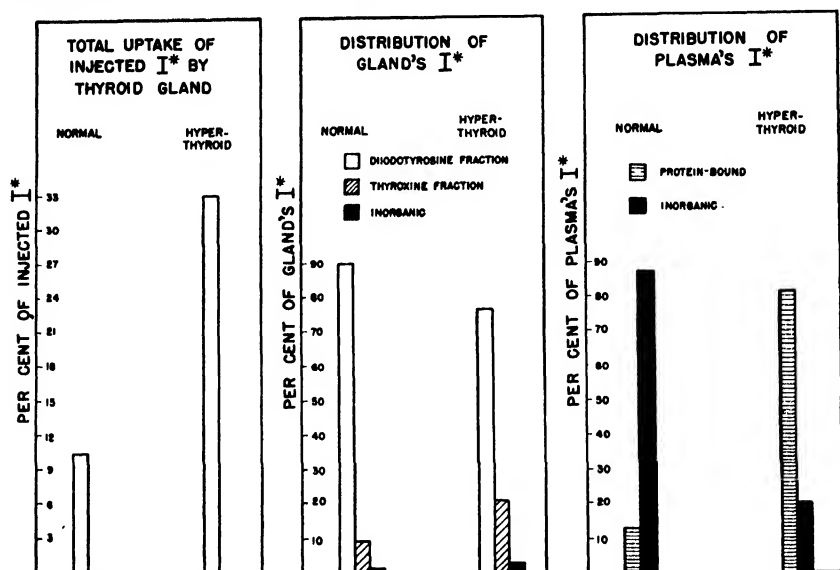


FIGURE 8. The uptake of  $I^*$  and its distribution in the thyroid and plasma of normal and thyrotropic hormone-injected guinea pigs. The measurements were made 16 hours after the intraperitoneal injection of a tracer dose of the  $I^*$ .

ence of the thyrotropic hormone, the normal distribution of the gland's  $I^*$  between thyroxine and diiodotyrosine is changed. In the hyperactive gland, a smaller percentage than normal of the gland's  $I^*$  was found as diiodotyrosine, whereas a larger percentage than normal was present as thyroxine.

**THE DISTRIBUTION OF  $I^*$  BETWEEN THYROXINE AND DIIODOTYROSINE IN PERIPHERAL TISSUES.** The striking effect of thyrotropic hormone on the metabolic fate of circulating inorganic iodide is shown in FIGURE 9, which depicts the distribution of the radioactive iodine contained in one of the peripheral tissues (in this case the small intestine) of guinea pigs at various time intervals after the injection of a tracer dose of radioactive iodine. It is assumed here that the movement of radio-iodine in and out of the tissues merely reflects the changes in radio-iodine concentration of the plasma.

Radioactive inorganic iodide disappeared more rapidly from the small intestine of the hyperactive than of the normal animal, a finding in keeping with the increased iodine-concentrating capacity of the thyroid gland of the thyrotropic hormone-injected guinea pig. This sharper drop in the inorganic iodide was accompanied by a sharper rise in the thyroxine-like  $I^*$ .

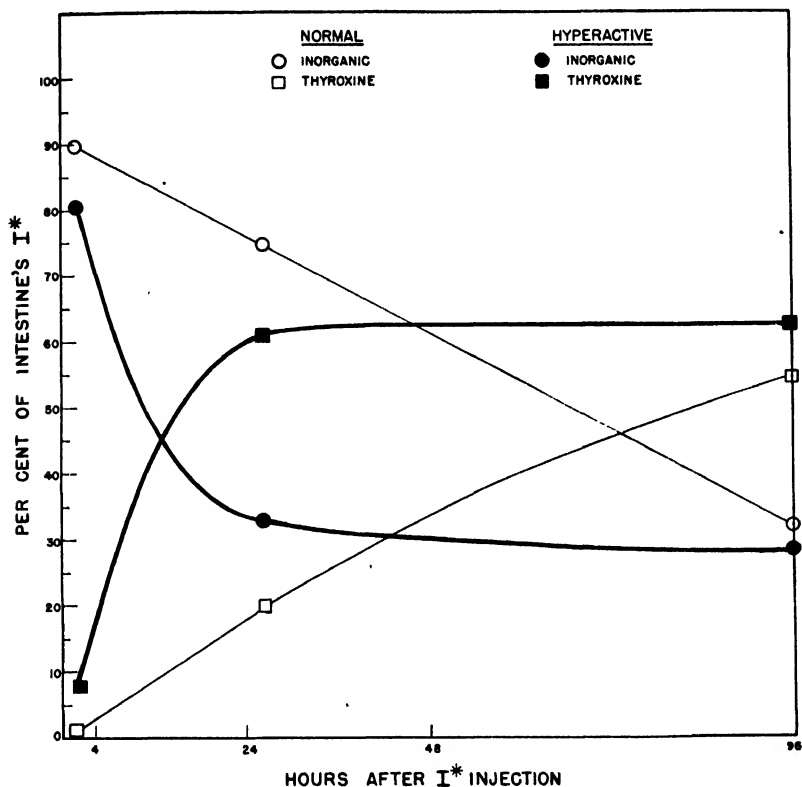


FIGURE 9. The distribution of the small intestine  $I^*$  in normal and thyrotropic hormone-injected guinea pigs.

It may be concluded from the experiments reported in this section that, in the thyrotropic hormone-treated animal, there is a more rapid removal of circulating radio-iodide by the thyroid gland, a more rapid conversion of this to thyroxine, and a more rapid release of radio-thyroxine by the gland into plasma and peripheral tissues.

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*Discussion of the Paper*

DR. WILBUR H. MILLER (*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut*):

My remarks are concerned with the question of the exchange reaction mentioned by Dr. Chaikoff. Some of us who have worked with diiodotyrosine have noticed the fact that this compound, under certain conditions, is quite unstable and can give off iodine in appreciable quantities. A somewhat detailed study of this problem has been published by Harington and Pitt Rivers.<sup>1</sup> Impetus for this study was furnished by the discovery of von Mutzenbecher<sup>2</sup> that diiodotyrosine on incubation in alkaline solution spontaneously gave rise to small quantities of thyroxine. This observation was confirmed by Block,<sup>3</sup> starting with synthetic diiodotyrosine. A theory for this reaction was proposed by Johnson and Tewkesbury,<sup>4</sup> who postulated hypoidite as the effective oxidizing agent. All of these observations point to the instability of this thyroxine precursor.

We have published<sup>5</sup> that diiodotyrosine undergoes an exchange reaction and, in view of such observations as above indicated, this is not too surprising. We divided the exchange behavior of diiodotyrosine into two parts: (1) the reactivity in the presence of iodine and (2) the reactivity in the presence of iodide.

In the presence of iodine in aqueous solution, the radioactivity passes either from the diiodotyrosine to the iodine, or *vice versa*, depending on the mol ratio of reactants and the compound with which the radioactive iodine atoms were associated originally. This reaction occurs readily at room temperature and the rate does vary with pH. We first observed this reaction when a thyroid tissue hydrolysate containing radio-diiodotyrosine was treated with potassium iodide-potassium iodate at around pH 2.

The exchange reaction between iodide and diiodotyrosine occurs much more slowly at room temperature, and to get a rate which is readily measured it is best to warm the reaction mixture. This exchange reaction is inhibited by compounds which react readily with iodine, such as thiouracil<sup>6</sup> and thiosulfate and is speeded up by very small quantities of elementary iodine. It is likely that the exchange between iodide and diiodotyrosine occurs only after some of the latter compound has decomposed with the liberation of sufficient iodine to allow this exchange to proceed. Heating would speed up both the decomposition rate and the exchange rate of diiodotyrosine.

<sup>1</sup> HARINGTON, C. R., & R. V. PITT RIVERS. *Biochem. J.* 39: 157. 1945.

<sup>2</sup> VON MUTZENBECHER, P. *Hoppe-Seyl. Z.* 261: 253. 1939.

<sup>3</sup> BLOCK, P., JR. *J. Biol. Chem.* 135: 51. 1940.

<sup>4</sup> JOHNSON, T. B., & G. B. TEWKESBURY. *Proc. Nat. Acad. Sci.* 28: 73. 1942.

<sup>5</sup> MILLER, W. H., G. W. ANDERSON, R. K. MADISON, & D. J. SALLEY. *Science* 100: 340. 1944.

<sup>6</sup> MILLER, W. H., R. O. ROBLIN, JR., & E. B. ASTWOOD. *J. Am. Chem. Soc.* 67: 2201. 1945.

These observations are offered merely to indicate again the need to insure that the results of any *in vivo* work are not modified by subsequent *in vitro* procedures during which such exchanges as have been observed above might occur.

DR. C. P. LEBLOND (*McGill University, Montreal, Canada*):

Dr. Chaikoff gave credit to Mann, Warren, and myself for publishing the first positive proof that diiodotyrosine was the precursor of thyroxine. The arguments, which we obtained from a study of the specific activity in the thyroids of dogs after administration of radio-iodine, were as follows:

(1) The specific activity of the thyroxine fraction, 48 hours after injection of radio-iodine, was greater than that of the iodide fraction at  $\frac{1}{2}$ , 8, or 48 hours and smaller than that of the diiodotyrosine fraction at these same time intervals. Consequently, diiodotyrosine was the only possible supplier of radio-iodine to thyroxine.

(2) This conclusion was in keeping with the gradual rise of the specific activity of thyroxine during the 48 hours after the injection, while the specific activity of diiodotyrosine remained steady throughout the whole period.

These results agree with the known facts of thyroxine chemistry. However, they were obtained with a small number of animals. Drs. Chaikoff and Taurog worked with a large number of animals and, by using Zilversmit's method for the analysis of the results, removed all possible doubt that Harington's theory on the synthesis of thyroxine from diiodotyrosine is correct.

# IODINE ABSORPTION AND UTILIZATION UNDER THE INFLUENCE OF CERTAIN GOITROGENS

By D. A. MCGINTY\*

*Research Laboratories, Parke, Davis and Company, Detroit, Michigan*

**A**BSORPTION of iodine by the thyroid gland, synthetic conversion of iodine into the various chemical forms in which it occurs there, and discharge of iodine from the gland involve mechanisms varying to a greater or lesser degree according to the extent to which other mechanisms are altered. The influence of dietary limitation of iodine intake on thyroid function has been investigated experimentally and is encountered often in clinical medicine. The effects of high iodine intake have been studied to a lesser extent, and it is the purpose of this communication to present experimental data bearing on this phase of thyroid function.

Experiments by Rawson (1944) and by Chaikoff (1944) and their respective co-workers, using a radio-iodine technique, have shown that administration of thiouracil induces an effective block to the uptake of iodine by the thyroid of the rat. Franklin, Chaikoff, and Lerner (1944), however, have demonstrated that surviving sheep thyroids do absorb iodide from a Ringer-bicarbonate medium containing thiouracil, although little or none of the absorbed iodine is converted into diiodotyrosine or thyroxine, as is the case in control experiments in which thiouracil is absent from the medium (Morton and Chaikoff, 1943). Differences between iodine-absorbing capacity of thyroids of intact thiouracil-treated animals and surviving thyroids respiring in a thiouracil-containing medium are not explained on the basis of iodine concentration in the environment.

We had observed, in our experiments dealing with quantitative evaluation of antithyroid activities of various chemical substances in rats, that the level of iodine intake during the experimental period influences not only the extent to which thyroid iodine concentration is reduced but the degree of hypertrophy of the gland as well. Astwood has reported that rats are more sensitive to thiouracil if maintained on low iodine intakes. He has demonstrated, furthermore (1944-45), that iodine is absorbed by the thyroid of the thiouracil-treated rat in amounts proportional to the daily intake. Vanderlaan and Bissell (1946) showed that such absorption occurs very rapidly in the chick as well as in the rat.

With these observations in mind, we have investigated quantitatively the amount of iodine absorbed when relatively large amounts of iodide

\* The author wishes to acknowledge his thanks and appreciation for the technical assistance of Mary L. Wilson and Virginia E. Hinchman.



were given to rats receiving thiouracil and other goitrogens. We became interested, at the same time, in the form in which iodine accumulated in the gland and whether or not the goitrogenic action of antithyroid compounds was modified with iodide administration.

### *Procedure*

Twenty-six- to twenty-eight-day female rats, weighing approximately 45 gm. at the start of the experiment, were used throughout. These were maintained at 25° C. in screen-bottom cages arranged for accurate recording of food and water intake.

Thiouracil and other antithyroid compounds were administered in a standard breeder ration containing yellow corn meal 33 parts, whole-wheat flour 31, whole-milk powder 21, linseed meal 7, brewer's yeast (Anheuser-Busch G) 3, alfalfa meal 2, dried hog liver 2,  $\text{CaCO}_3$  0.5, and C.P. NaCl 0.5. The daily intake of iodine from this diet varied between 0.7 and 1.4 micrograms, depending on the amount of food eaten. Additional iodine was administered as KI dissolved in the drinking water, which itself was iodine-free.

At the end of the experimental period, groups of 3–10 rats, similarly treated, were killed with chloroform, their thyroids removed, pooled, and weighed. They were analyzed for total iodine after alkali fusion by the method of Astwood and Bissell (1944).

In the thyroid fractionation experiments, no attempt was made to determine inorganic, thyroxine, and diiodotyrosine iodine according to current procedures. However, glands were fractionated with  $\text{Zn(OH)}_2$ , the precipitate being considered as protein-bound iodine and the supernatant as non-protein-bound iodine. Pooled glands were homogenized in a mortar with distilled water, transferred to a 15-cc. centrifuge tube and made up to 4–5 cc. volume. After addition of 0.25 cc. each of  $\text{Zn(SO}_4)_2$  and NaOH solutions (Somogyi), the contents of the tubes were mixed and centrifuged. The precipitate was washed three times with 3–4 cc. portions of filtered  $\text{Zn(OH)}_2$  solution and analyzed for iodine. The supernatant from the centrifuged  $\text{Zn(OH)}_2$  precipitate and washings were analyzed separately.

### *Results and Discussion*

FIGURE 1 shows the effect of increasing intakes of iodide on total thyroid iodine concentration and on thyroid weight of rats receiving 4–5 mg. thiouracil daily for a period of 10 days. With increasing intakes, iodine content of the thyroid ranged from 0–2 mg. per cent when the daily iodine intake was 8–10 micrograms, to values of over 40 mg. per cent when 10 milligrams of KI were consumed daily. At the end of the 10-day period, total iodine of the gland at the 10-microgram KI intake level was .10–.15  $\mu\text{g.}$ , whereas at the high iodide intake of 10 milligrams, thyroid iodine reached 4–6  $\mu\text{g.}$  per rat. That this uptake was not due to simple diffusion of iodide into the thyroid from abnormally high blood

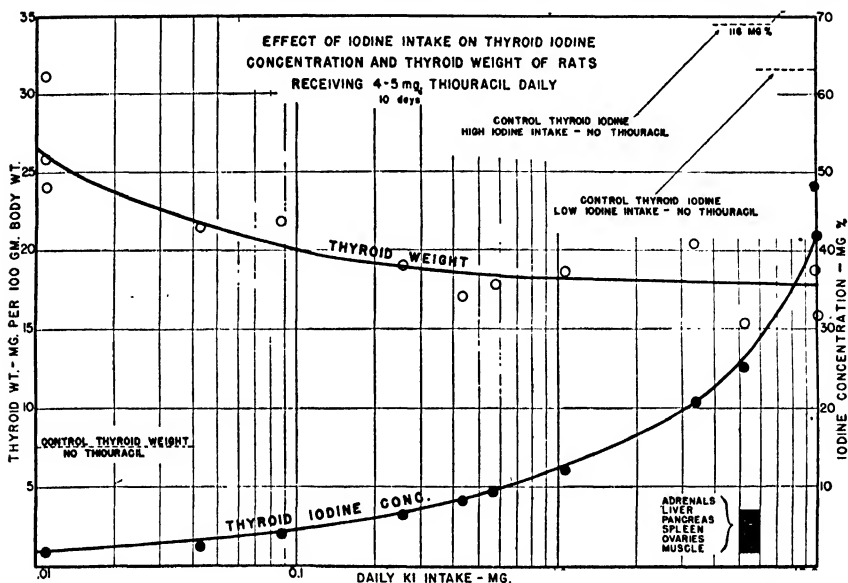


FIGURE 1

concentration is shown by the relatively low level of iodine in several other tissues at KI intake of 5-6 mg. daily. Attention is called to the levels of thyroid iodine concentration at low and high iodine intakes in control groups receiving no thiouracil in their diet.

The goitrogenic action of thiouracil is appreciably reduced with high iodine intakes. This is not so pronounced in the 10-day experiments as in those in which treatment was carried out for longer periods. Antagonism of iodide toward this effect of thiouracil is not due to a general toxic action, since growth rate of rats on thiouracil alone, iodide alone or a combination of the two in dosages employed was similar over the experimental period to that of untreated controls.

When thyroid glands from rats treated with thiouracil and iodide were fractionated with  $\text{Zn}(\text{OH})_2$ , nearly all of the iodine was found in supernatants indicating its non-protein-bound character. This is illustrated in TABLE 1, in another experiment in which two groups of thiouracil-treated rats were given 10-11 micrograms and 9-10 milligrams of iodine respectively, in the drinking water. The bulk of the iodine was found in the non-protein-bound fraction, in contrast to the corresponding control group receiving no thiouracil, in which most of the iodine remained in the protein-bound fraction. It is quite evident, from these results, that the primary effect of thiouracil in causing loss of protein-bound iodine from the thyroid is not interfered with by iodide administration, and that iodine fixation in the gland with high intakes involves an independent mechanism.

These experiments were repeated in rats receiving maximal doses of

TABLE 1

THIOURACIL-IODINE ADMINISTRATION  
(10 rats per group --10 days treatment)

Diet supplement	Av. body wt. gain, gm. per day	Av. daily iodine intake per 100-gm. rat	Thyroid wt. mg./100-gm. rat	Thyroid iodine mg. %	
				$\overbrace{\text{Zn(OH)}_2}^{\text{Ppt.}}$	$\overbrace{\text{Zn(OH)}_2}^{\text{Supt.}}$
.1% Thiouracil + 1 mg. KI/liter H <sub>2</sub> O	} 3.2	11 $\mu$ g.	30.8	0.1	0.3
.1% Thiouracil + 1000 mg. KI/liter H <sub>2</sub> O					
Control + 1 mg. KI/liter H <sub>2</sub> O	} 4.0	10 $\mu$ g.	6.7	62.2	1.4
Control + 1000 mg. KI/liter H <sub>2</sub> O					
	} 3.4	9.7 mg.	7.2	95.6	3.0

thiouracil and other goitrogens for 25 days, followed by a similar period of combined treatment with goitrogen and high iodine. This was done in order to observe the effect of high iodine intake on iodine content of the thyroid and on goitrogenic action of the drug subsequent to depletion of iodine content by preliminary treatment with antithyroid compound.

Results are shown in TABLE 2. After a 25-day period of treatment with thiouracil, 5-aminothiadiazole-2-thiol (TC-68), 3-(phenylaminomethyl)-thiazolidine-2-thione (TC-105), KSCN, and propyl thiouracil, when the iodine content of the thyroid has been reduced to minimal levels, high iodine intake causes a reaccumulation of iodine in the gland. This is found, however, in the  $\text{Zn(OH)}_2$ -soluble fraction, as was the case in those experiments in which antithyroid compound and iodide were given together at the beginning of the experiment. It will be observed, also, that there occurred a significant reduction in thyroid weight in three instances after the initial treatment with antithyroid substance, and that, in the other instance, no further increase occurred when iodine was added. Again, examination of data on body weight change, or food and water consumption, indicated that this antigoitrogenic effect of iodide was not due to any toxic effect *per se*.

KSCN at a concentration of .2% in the diet caused a reduction of thyroid iodine content to 9.3 mg. per cent after 51 days on the low iodine intake of 1.4  $\mu$ g. daily. Most of the iodine left in the gland was in the  $\text{Zn(OH)}_2$  precipitate fraction, and only a slight enlargement of the thyroid occurred. Introduction of substantial amounts of iodine to the diet at the end of 25 days caused restoration of iodine to the thyroid and disappearance of goiter. In contrast to the blocking action of thiouracil and other potent goitrogens, KSCN is quite unable to prevent synthesis of protein-bound iodine when sufficient iodine is taken in the diet. It may be observed, however, that substantially more non-protein-bound

TABLE 2

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low Iodine Diet Containing Thiouracil and Other Goitrogens for 25 Days, Followed by a 26-Day Period during which the Goitrogens were Administered Simultaneously with a High Iodine Intake. 3-6 Rats per Group. Thyroid Weight and Iodine Concentrations from Pooled Thyroids of Each Group

Exp. No.	Goitrogen	Conc. in food	Body wt. gain gm./day	At. daily iodine intake per 100-gm. rat	Thyroid wt. mg./100-gm. rat	Thyroid iodine mg. % $\left\{ \begin{array}{l} \text{Zn(OH)}_2 \\ \text{Ppt.} \end{array} \right.$	Thyroid iodine mg. % $\left\{ \begin{array}{l} \text{Zn(OH)}_2 \\ \text{Supt.} \end{array} \right.$	Duration of experiment, days
1422	Thiouracil	.1%	2.2	1.1 $\mu$ g.	51.9	0.0	0.1	25
1252			1.4	1.0 $\mu$ g.	70.2	0.6	1.6	25+26
1253			1.6	8.3 mg.	36.9	1.6	55.0	
1423	TC-68*	.1%	2.2	1.1 $\mu$ g.	59.2	0.0	0.0	25
1260			1.5	1.1 $\mu$ g.	67.0	1.1	0.7	25+26
1261			1.6	12.8 mg.	38.4	1.0	45.6	
1424	TC-105†	.1%	2.1	0.8 $\mu$ g.	57.1	0.0	0.0	25
1267			1.4	0.9 $\mu$ g.	74.5	0.6	1.4	25+26
1268			1.3	15.7 mg.	57.0	0.8	100.5	
1511-2	6-n-Propyl thiouracil	.03%*	2.4	1.1 $\mu$ g.	60.4	0.3	0.7	25
1523-4			1.4	1.1 $\mu$ g.	93.1	0.3	2.0	25+26
1525-6			1.4	10.5 mg.	49.2	0.6	43.0	
1274	KSCN	.2%	2.1	1.4 $\mu$ g.	9.3	7.2	2.1	25+26
1275			2.0	10.0 mg.	7.7	127.3	11.8	
1513-4	Controls		3.1	1.4 $\mu$ g.	8.2	58.2	4.2	25
1527-8			2.5	1.4 $\mu$ g.	6.9	30.8	2.8	25+26
1529-30			2.6	11.3 mg.	7.4	115.0	5.2	

\* 5-Aminothiazole-2-thiol.

† 3-(Phenylaminomethyl)-thiazolidine-2-thione.

iodine was found in the KSCN-treated rats receiving high iodine than in the corresponding control group. These results, as a whole, are consistent with those of other investigators who have found that KSCN is goitrogenic only when iodine intake is below certain minimal levels.

The rats treated with effective antithyroid compounds gained less weight than did the controls. The decrease in weight gain was not evident until after two to three weeks of treatment with the drug. Therefore, we would contribute this decreased weight gain to the development of an athyreotic state rather than to a toxic effect of the drug given.

We also find that recovery from the effects of thiouracil and other goitrogens is accelerated by a high iodine intake. This is shown in a limited experiment in which rats were treated for 10 days with a low iodine diet containing .1 per cent thiouracil followed by a 14-day period during which no thiouracil was given and iodine intake was increased. The degree of restoration of protein-bound iodine and recovery from goiter is shown in TABLE 3. It may be noted that even at a low intake of 1.4  $\mu$ g. iodine daily some absorption and fixation of iodine into the thyroid occurred and considerable reduction in thyroid weight took place. It appears that avidity of the gland for iodine increases as its content diminishes.

The effect of diiodotyrosine administration on the action of thiouracil is shown in TABLES 4 and 5. In the first experiment, diiodotyrosine at a concentration of .1 per cent in the food was administered simultaneously with thiouracil for a period of 10 days. The results are similar to those obtained with iodide at corresponding iodine intakes. Since no attempt was made to determine the diiodotyrosine content of the thyroid glands at the end of the experiment, one cannot state with certainty whether accumulation of iodine in the supernatant fraction under thiouracil treatment was diiodotyrosine itself, which is soluble in  $\text{Zn(OH)}_2$ , or iodide which formed by decomposition of diiodotyrosine. There is no evidence that diiodotyrosine decomposition did not occur. The antigoitrogenic effect of diiodotyrosine is similar to that obtained with iodide at corresponding intakes. Dempsey and Astwood (1943) have suggested that the antigoitrogenic action of diiodotyrosine may be due to traces of thyroxine in the compound or formed from it by oxidation. We have found that diiodotyrosine administered in the drinking water is much more antigoitrogenic than when administered in the food, particularly when solutions were prepared at infrequent intervals. These results lend support to the view that thyroxine is formed under these circumstances. In the next experiment, diiodotyrosine was incorporated into the diet subsequent to a 10-day period of thiouracil treatment. Results are shown in TABLE 5. When thiouracil is continued for 14 days after the preliminary 10-day treatment, diiodotyrosine or iodide accumulates in the non-protein-bound fraction. If thiouracil is discontinued and diiodotyrosine administered for 14 days, iodine is found mainly in the  $\text{Zn(OH)}_2$  precipitate fraction. Here, again, uncertainty exists as to whether diiodo-

TABLE 3

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low Iodine Diet Containing Thiouracil for 10 Days, Followed by a 14-Day Period during which Thiouracil is Discontinued and a High Iodine Substituted for the Low Iodine Intake. Six Rats per Group. Thyroid Weight and Iodine Concentrations from Pooled Thyroids of Each Group									
Exp. No.	Goitrogen	Conc. in food	Body wt. gain, gm.	Avg. daily iodine intake per 100-gm. rat	Thyroid wt. rat	Thyroid iodine mg. per 100-gm.	Thyroid iodine mg. %		Duration of experiment, days
							$\overbrace{\text{Zn(OH)}_2}^{\text{ppt.}}$	$\overbrace{\text{Zn(OH)}_2}^{\text{supt.}}$	
Average	Thiouracil	.1%	3.2	1.1 $\mu\text{g.}$	33.5	0.3	0.3	1.0	10
1509-10	Thiouracil	.1%	3.3	1.4 $\mu\text{g.}$	10.7	26.6	26.6	1.4	10+14
1515-16	Thiouracil	.1%	3.1	13.0 $\mu\text{g.}$	8.4	37.8	37.8	1.5	10+14
1517-18	Thiouracil	.1%	2.8	15.7 $\mu\text{g.}$	8.4	68.4	68.4	6.5	10+14

TABLE 4

## THIOURACIL-DIIODOTYROSINE ADMINISTRATION

(Six rats per group—10 days treatment; daily iodine intake from food—0.7 to 1.2  $\mu\text{g.}$ )

Diet supplement	Av. body wt. gain—gm. per day	Thyroid wt. mg./100-gm. rat	Thyroid iodine mg. %	
			$\text{Zn(OH)}_2$ Ppt.	$\text{Zn(OH)}_2$ Supt.
Thiouracil .1%	3.1	32.6	0.8	0.4
Thiouracil .1% + diiodotyrosine .1%	3.0	29.5	0.0	78.2
	3.1	22.6	5.2	51.6
	3.4	20.7	3.6	45.0
	2.4	19.2	1.6	49.0
Diiodotyrosine .1%	3.0	8.6	98.4	14.6
	2.2	8.9	109.0	10.2
	3.4	6.6	71.8	14.5
Controls	3.2	6.7	42.6	1.4

TABLE 5

## THIOURACIL-DIIODOTYROSINE ADMINISTRATION

(Six rats per group—24 days total treatment;  
av. daily iodine intake from food—1.0 to 1.2  $\mu\text{g.}$ )

.1% Thiouracil for 10 days, followed by 14 days' treat- ment with	Av. body wt. gain—gm.	Thyroid wt. mg./100-gm. rat	Thyroid iodine mg. %	
			$\text{Zn(OH)}_2$ Ppt.	$\text{Zn(OH)}_2$ Supt.
.1% Thiouracil—10 days	31	32.6	0.4	1.0
.1% Thiouracil	56	51.9	0.1	0.0
.1% Thiouracil + .1% diiodotyrosine }	55	36.5	0.4	77.8
.1% Diiodotyrosine	68	19.5	28.1	2.9
Controls (24 days)	85	6.5	76.9	2.2

tyrosine survives in tissues as such or whether results are due to liberated iodide.

In a final series of experiments, thiouracil was administered to rats for 24 days, during the last 14 of which they received thyroxine in varying doses. Observations were made on thyroid weight and  $\text{Zn(OH)}_2$ -precipitable and non-precipitable iodine values of the glands.

Dempsey and Astwood (1943) and Reincke, Mixner, and Turner (1945) have shown that thyroxine in adequate amounts given concurrently with thiouracil will prevent goiter, loss of thyroid iodine, and lowering of metabolic rate. Our own data, in substantial agreement with those of Astwood and Bissell, indicate that three micrograms of *dl*-thyroxine, given subcutaneously once daily, will prevent any thyroid enlargement in thiouracil-treated rats over a period of 10 days, whereas to maintain a normal thyroid iodine concentration under the same circumstances 20–30  $\mu\text{g.}$  are required. At this dose of thyroxine, thyroid weight was somewhat diminished from normal, suggestive of partial pituitary inhibition.

Objectives of the experiment were two-fold: first, to determine whether thiouracil would prevent collection and storage of administered thyroxine when given in excessively large doses, and, second, to determine what dose of thyroxine given an animal already goitrous and continuing to receive thiouracil would produce involution of the hyperplastic gland. Rats were maintained on thiouracil, 0.1 per cent in the diet, for 24 days. Beginning on the 11th day, the animals were given thyroxine daily by subcutaneous injection in doses of 18, 40, and 80  $\mu$ g. In another experiment, desiccated thyroid in a concentration of .2 per cent in the food was administered on the 11th day. Only in the latter case and in one experiment with the 80  $\mu$ g. dose of thyroxine was there evidence of toxicity from these substances. At the 18  $\mu$ g. dose level, one series was done with coadministered iodide, another without added iodine. In the 40  $\mu$ g. thyroxine experiment, small amounts of iodide were given throughout. None was added to the diet in the case of the 80  $\mu$ g. dose of thyroxine nor during administration of thyroid orally.

Results are recorded in TABLE 6. The first two experiments are controls, one showing the concentration and distribution of iodine in the thyroid and thyroid weight of rats treated for 10 days with thiouracil alone, the other with coadministered thyroxine, 18 micrograms daily. Experiment 1422 shows the results following administration of thiouracil for 24 days. When thyroxine is injected beginning on the eleventh day of thiouracil treatment and continued until the 24th day, it may be seen that at none of the dose levels of thyroxine nor when the rats were receiving thyroid orally was there any accumulation of iodine in the thyroid except for a small increase in concentration with 80  $\mu$ g. thyroxine and with 45  $\mu$ g. *l*-thyroxine equivalent from oral thyroid. Failure to absorb or utilize iodine may be due either to the presence of thiouracil or to an inhibited state of the pituitary from excessive concentrations of circulating thyroxine. It has been suggested that thyrotropic hormone is as essential for thyroid synthesis as it is for discharge of hormone from the gland. It does not follow, however, that the inability of the thyroid to absorb thyroxine itself is due to the presence of thiouracil, since there is no evidence that this substance is absorbed by the gland under conditions when thiouracil is absent. These observations are in conformity with those of Astwood and Bissell, who employed the smaller doses of thyroxine in similar experiments.

To examine further the quantitative aspect of inhibitory effects of thyroxine on pituitary thyrotropic activity, two experiments were done. Thiouracil was administered for 10 days, then discontinued and followed immediately by 14 days' treatment with thyroxine alone. At a daily dose of 18  $\mu$ g., iodine accumulated in the thyroid in a concentration comparable to that observed in control rats receiving no thyroxine. There is, in fact, not only no evidence of pituitary inhibition but an apparent acceleration of rate of iodine storage at this dose of thyroxine, especially when the two rat groups are compared at similar intakes of iodide.



TABLE 6

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low and Moderate Iodine Diet Containing Thiouracil for 10 Days, Followed by a 14-Day Period during which Thiouracil was Administered Simultaneously with Thyroxine or during which Thiouracil was Discontinued and Thyroxine Given Alone. Six Rats per Group. Thyroid Weight and Iodine Concentration Estimated from Pooled Thyroids of Each Group

Exp. No.	Diet supplement	Body wt. gain gm./day	Avg. daily iodine intake* per 100-gm. rat	Thyroid wt. mg./100-gm. rat	Thyroid iodine mg. % $\left\{ \begin{array}{l} \text{Zn(OH)}_2 \\ \text{Ppt.} \end{array} \right.$ $\left\{ \begin{array}{l} \text{Zn(OH)}_2 \\ \text{Suppl.} \end{array} \right.$	
Average	Thiouracil .1%—10 days	3.2	1.1 $\mu\text{g.}$	33.5	0.3	1.0
Average	$\left\{ \begin{array}{l} \text{Thiouracil .1\% + thyroxine} \\ \text{18 } \mu\text{g. daily—10 days} \end{array} \right\}$	2.8	10.3 $\mu\text{g.}$	6.0	60.0	3.7
1422	Thiouracil .1%—24 days	2.3	1.1 $\mu\text{g.}$	51.9	0.0	0.1
1750-51	Thiouracil .1%—24 days	2.7	1.1 $\mu\text{g.}$	50.7	0.6	0.6
1754-55	Thyroxine 18 $\mu\text{g.}$ —last 14 days	2.6	10.3 $\mu\text{g.}$	67.0	1.2	1.1
1798-99	$\left\{ \begin{array}{l} \text{Thiouracil .1\%—24 days} \\ \text{Thyroxine 40 } \mu\text{g.} \text{—last 14 days} \end{array} \right\}$	2.7	9.9 $\mu\text{g.}$	29.4	0.9	1.2
1868-69	Thiouracil .1%—24 days	2.5	1.0 $\mu\text{g.}$	13.5	2.4	1.1
1872-73	Thyroxine 80 $\mu\text{g.}$ —last 14 days	1.7	1.2 $\mu\text{g.}$	9.6	3.5	1.3
1849-50	$\left\{ \begin{array}{l} \text{Thiouracil .1\%—24 days} \\ \text{Thyroid .2\% (45 } \mu\text{g. thyx.)—} \\ \text{last 14 days} \end{array} \right\}$	1.2	1.2 $\mu\text{g.}$	11.0	3.7	2.1
1509-10	$\left\{ \begin{array}{l} \text{Thiouracil .1\%—10 days} \\ \text{Autopsy 14 days later} \end{array} \right\}$	3.3	10.7 $\mu\text{g.}$	10.7	26.6	1.4
1752-53	Thiouracil .1%—10 days	2.8	1.1 $\mu\text{g.}$	11.6	33.1	2.8
1756-57	Thyroxine 18 $\mu\text{g.}$ —14 days	2.9	11.1 $\mu\text{g.}$	11.5	41.7	2.9
1800-01	Thiouracil .1%—10 days	2.1	9.3 $\mu\text{g.}$	20.5	6.1	1.1
1870-71	Thyroxine 40 $\mu\text{g.}$ —14 days	2.0	1.3 $\mu\text{g.}$	9.6	1.8	0.9
1851-52	$\left\{ \begin{array}{l} \text{Thiouracil .1\%—10 days} \\ \text{Thyroid .2\% (50 } \mu\text{g. thyx.)—} \\ \text{last 14 days} \end{array} \right\}$	1.7	1.2 $\mu\text{g.}$	12.4	26.3	1.4

\* Does not include iodine from injected thyroxine.

In contrast, when thyroxine is administered in doses of 40  $\mu$ g. daily following thiouracil treatment, little or no iodine accumulates in the thyroid. This result may be interpreted as indicating pituitary inhibition and failure of thyrotropin formation, whereas the 18  $\mu$ g. dose was insufficient to produce this effect. In this connection, it may be recalled that Astwood showed that rat thyroids depleted of iodine by thiouracil did not accumulate iodine in appreciable amounts after hypophysectomy.

In a single experiment conducted with oral thyroid instead of thyroxine, no interference with iodine absorption or synthesis into protein-bound form was evident. This occurred despite an estimated absorption of the equivalent of 50  $\mu$ g. *L*-thyroxine daily.

These observations may be summarized as follows: Administration of large doses of iodide to rats treated chronically with thiouracil, propyl thiouracil, and other antithyroid compounds having similar mode of action results in absorption of iodine by the thyroid, proportional over a wide range to the amount of iodide ingested daily.

Iodine so absorbed is not synthesized into protein-bound form but remains in  $\text{Zn}(\text{OH})_2$  supernatant fractions made from homogenized thyroid tissue. The form in which it exists under such circumstances is not known. Salter's (1945) suggestion that a loose combination exists between iodide and protein colloid, probably adsorptive in character, is logical. Astwood (1944-45) suggests that such a mechanism of iodide absorption and accumulation exists independently of the iodination system in the gland and that only the latter is inhibited by thiouracil.

The goitrogenic action of thiouracil and other antithyroid compounds is appreciably reduced by high iodine intakes. This action is due, we believe, not to any toxic effect of iodide but to diminished sensitivity of the thyroid cells to thyrotropic stimulation. Recovery from the goitrogenic action of thiouracil and restoration of protein-bound iodine are accelerated by high iodine intakes. These latter observations are in conformity with recent results reported by Taurog and Chaikoff (1946), showing that high iodine intakes led to increased concentrations of thyroxine iodine in rat thyroids. In agreement with the observations of Astwood, Salter, Chaikoff, and others, we find that the goitrogenic and antithyroid action of KSCN is readily inhibited by iodide even at relatively low intakes.

Diiodotyrosine in high doses is partially inhibitory to the goitrogenic action of thiouracil and, like iodide, accumulates in the non-protein-bound fraction of the thyroid when administered with thiouracil. Having no experimental evidence one way or the other at this time, we would favor Salter's view that these results are due to iodide formed from decomposition of diiodotyrosine rather than to diiodotyrosine itself.

*dl*-Thyroxine in daily subcutaneous injections of 3-4 micrograms given concurrently with thiouracil will prevent thyroid hypertrophy over a test period of 10 days. However, with this dose of thyroxine, iodine con-

centration of the thyroid diminishes, indicating either that pituitary thyrotropic action resulting from thiouracil treatment is inhibited only in part, or that hormonal iodine leaves the gland despite continuous addition to the body of amounts of hormone believed adequate for metabolic requirements. A daily dose of 20–30  $\mu\text{g}$ . thyroxine administered concurrently with thiouracil will not only inhibit goiter but prevent loss of thyroid iodine as well.

Administration of *dl*-thyroxine to rats whose thyroids have been depleted of iodine by thiouracil and who continue to receive thiouracil is followed by involution of the hyperplastic gland with doses of 40 and 80  $\mu\text{g}$ . daily but not with 18  $\mu\text{g}$ . Oral thyroid was effective also in doses equivalent to 45  $\mu\text{g}$ . *L*-thyroxine. In no case, however, was there any restoration of iodine to the thyroid either in non-protein-bound form from dietary iodine or in organic form from administered thyroxine. Failure of iodine storage by the thyroid may be due in part to the blocking action of thiouracil at the thyroid, and in part to thyroxine-induced inhibition of thyrotropin secretion at the pituitary. The latter reasoning is borne out by the observation that recovery from thiouracil is inhibited by 40  $\mu\text{g}$ . thyroxine daily, whereas the smaller dose of 18  $\mu\text{g}$ . appears to be without effect on the iodine absorption and iodine synthesis mechanism.

With reference to the blocking action of thiouracil on iodine absorption by the thyroid, emphasis should be placed on the conditions under which this takes place. Experimental work already cited indicates that iodine enters the thyroid gland freely when intake is high and when concentration in the medium bathing the thyroid is above certain critical levels. As has been pointed out, iodine absorbed under such circumstances remains in a non-protein-bound form as long as thiouracil or thiouracil-like conditions prevail.

If, however, iodine concentration in the environment is below certain critical levels, thiouracil prevents absorption by the thyroid. Observations of Rawson, Tannheimer, and Peacock (1944) and Franklin, Lerner, and Chaikoff (1944) in rats, as well as of Larson, Keating, Peacock, and Rawson (1945) in chicks, showed that administration of thiouracil orally for several days results in a marked reduction in the iodine-collecting capacity of the thyroid from injected radioactive sodium iodide. They also demonstrated that single subcutaneous injections of 5 milligrams of thiouracil suspension completely inhibit collection of radio-iodine by the chick thyroid within one hour after injection. These investigators showed, furthermore, that the degree of inhibition to radio-iodine collection diminished with decreasing amounts of thiouracil, varying as a linear function of the dose. Recovery from iodine absorption block induced by chronic thiouracil feeding or after single injections of the drug varies between the rat and chick experiments, but is generally complete in 24 hours. Total iodine administered as carrier with radio-iodine was uniformly 2  $\mu\text{g}$ ., an amount which, while it undoubtedly raised blood

concentration, was insufficient to cause iodine absorption by the mechanism described earlier in this communication.

We have employed this technique as a means of evaluating quantitatively antithyroid properties of other goitrogenic substances both as to their immediate effectiveness and as to their duration of action. However, we wish to report only such details of these experiments as are pertinent to the immediate subject under consideration, namely, that iodine block at the thyroid does occur and that it involves a mechanism common apparently to all of the goitrogenic substances studied. In these experiments, radio-iodine collection by the thyroid was determined in rats and chicks following single subcutaneous injections of drug suspensions in varying amounts. This research was carried out in collaboration with Dr. R. W. Rawson and will be reported in detail jointly. Radio-iodine was injected one hour, eight hours, 24 hours and, in some cases, 48 hours after administration of the compound under study. The compounds investigated included thiouracil, propyl thiouracil, benzyl thiouracil, TC-68, TC-105, 2-aminothiazole, KSCN, and methyl thiouracil. Selection was based in part on laboratory experience with these compounds, and the compounds were also chosen because they are representative of a considerable range of antithyroid potency as judged from chronic feeding tests.

*Procedure.* In order to differentiate between iodine-blocking activities of the compounds studied and to exaggerate differences between dosages, iodine-absorbing capacity by the thyroid of rats used in these experiments was increased by maintaining the animals on a low iodine diet for several weeks prior to injection of the goitrogen. The diet used was the one described earlier. This was slightly goitrogenic over the pre-treatment period when compared with the same diet containing iodized NaCl. It was, however, quite adequate for normal growth.

Following the preliminary low iodine dietary period, rats were divided into groups of 8-11 each. Antithyroid compounds were injected subcutaneously as suspensions in 10 per cent acacia solution. At designated intervals thereafter, one cubic centimeter of a NaI solution containing two micrograms of iodine and two microcuries of  $I^{131}$  was injected intraperitoneally. Uniformly at four-hour periods following radio-iodine injection, rats of each group were killed with chloroform, thyroids were removed, weighed, and transferred to vials containing dilute NaOH for solution. Results are expressed as per cent of collection by thyroids of control rats not treated with goitrogen but given radio-iodine at the same time.

One- to two-day-old sex-linked cockerels were given one guinea pig unit thyrotropic hormone daily for four days. On the fifth day, the chicks in groups of ten were injected subcutaneously with aqueous suspensions of antithyroid compounds in varying amounts. One hour later, 2.0 microcuries  $I^{131}$  in 1.0 cc. of a solution of sodium iodide containing 2 micro-

grams iodine were injected subcutaneously. Chicks were killed four hours later. Results are expressed, as in the case of the rat experiments, as per cent of control uptake.

*Results.* Only the data on radio-iodine collection one hour following administration of goitrogen will be presented here. FIGURE 2 shows the

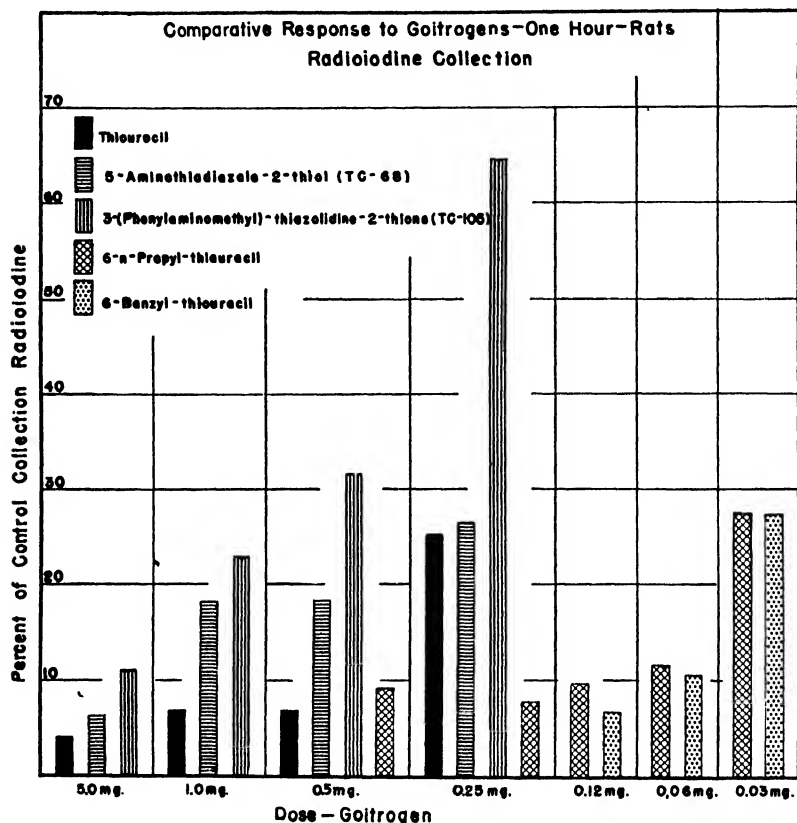


FIGURE 2

results for thiouracil, TC-68, TC-105, propyl thiouracil, and benzyl thiouracil in the rat. Arbitrarily, radio-iodine collection below 12 per cent of that of control groups was considered to be an effective block, and examination of the diagram shows that the minimal dose which will induce such a block in the doses used is 0.5 mg. for thiouracil, 5.0 mg. for TC-68 and TC-105, and .06 mg. for propyl and benzyl thiouracil. The corresponding value for methyl thiouracil, not shown on the chart, was 0.3 mg., showing that its iodine-blocking potency one hour following injection was almost twice that of thiouracil, despite the fact that in chronic tests for activity it has about the same goitrogenic activity as thiouracil. The low activity of TC-68 and TC-105, shown in these experiments,

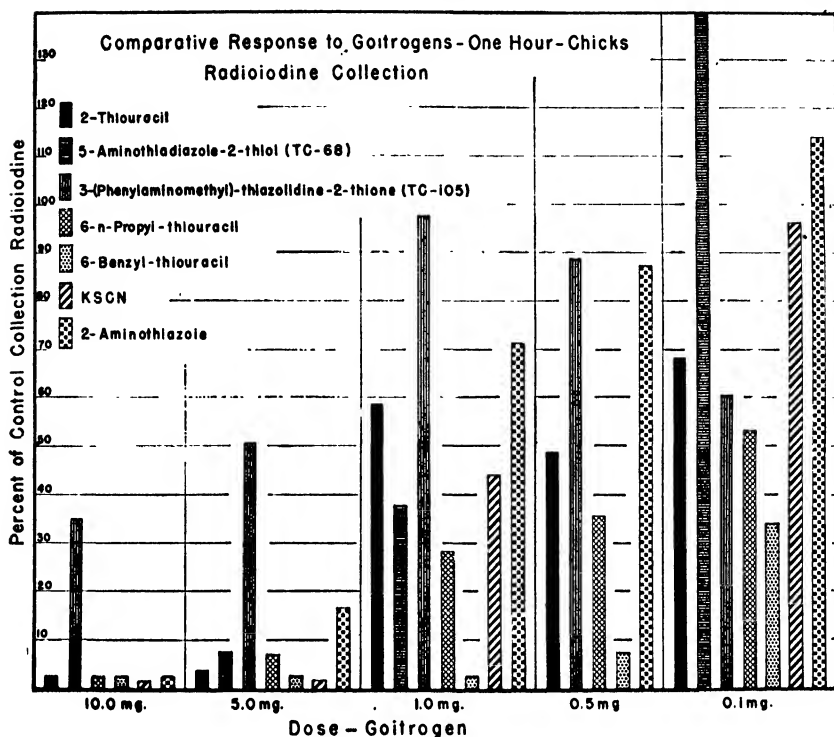


FIGURE 3

may also be contrasted with their relatively high goitrogenic activity in the chronic tests.

FIGURE 3 shows iodine collection figures for thiouracil, TC-68, TC-105, propyl and benzyl thiouracil, aminothiazole, and KSCN at various levels in chicks one hour after administration of the drug. With all compounds except TC-105, 10-mg. doses are effective in inducing an almost complete block to iodine absorption by the thyroid gland. At the 10-mg. dose, TC-105 is only weakly effective. Aminothiazole at 5 mg., while interfering considerably with iodine absorption, does not produce an effective block. This conforms with its comparatively low activity in chronic tests in rats, that is, about 15 per cent of that of thiouracil. Thiouracil, TC-68, propyl and benzyl thiouracil, and KSCN are effective at 5-mg. doses. At doses of 1.0 mg. and 0.5 mg., only benzyl thiouracil prevents absorption of injected radio-iodine in the one-hour period.

It is not the purpose here to discuss the relative potencies of these antithyroid substances as estimated by this test nor to compare the values obtained with those procured by other means. These experiments are presented only to illustrate that, when circulating iodide concentration is low, the goitrogens uniformly block uptake by the thyroid. This effect occurs within minutes after administration and persists for several hours,

depending to some extent on the dose. The mechanism of block is yet to be clarified, with a possible exception in the case of KSCN, which is believed to be due either to the formation of a stable iodine-KSCN chemical combination from which iodine cannot be freed or to interference with iodine absorption by its mass effect at the cell membrane.

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### *Discussion of the Paper*

DR. E. D. GOLDSMITH (*New York University, New York, N. Y.*):

In our paper with Dr. Keston, we reported that analysis of the thyroid glands of thiourea-treated rats, 48 hours after the injection of radioactive iodine, revealed no appreciable amounts of radioactive iodine in any form, whereas thyroid glands from normal rats contained considerable quantities of radioactive iodine, chiefly in organic combination. However, we had also observed (unpublished) that, shortly after administration, large amounts of radioactive iodine could be detected in the thyroids of thiourea-treated animals. *In vitro* experiments disclosed that hyperplastic thyroids from rats receiving thiourea in their ration collected large amounts of iodide, although relatively little organically bound iodine was formed. It would thus appear that iodine uptake is not inhibited by thiourea but that it passes through the gland rapidly and does not enter into organic combination.

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# MECHANISMS OF ACTION OF VARIOUS ANTITHYROID COMPOUNDS

By E. B. ASTWOOD

*Joseph H. Pratt Diagnostic Hospital, and Department of Medicine, Tufts College  
Medical School, Boston, Massachusetts*

AS long as we do not know just how thyroid function is controlled, we cannot define accurately how that function is inhibited. The production and secretion of thyroid hormone in the normal individual is nicely regulated by mechanisms, the nature of which we are only partially aware. It is possible, however, that a fuller understanding of normal thyroid function will result from a study of disturbances of thyroid activity and from an analysis of the possible mechanism of action of agencies which interfere with the normal process. The synthesis of thyroid hormone may be regarded as the primary function of the thyroid gland. Subsidiary to this are the storage of hormone within the gland and its release into the blood stream in appropriate quantities. These functions are dependent upon the complex cellular activities of the thyroid parenchyma, which, in turn, are regulated by the other influences, including the thyrotropic activity of the anterior pituitary.

An attempt will be made, in this review, to analyze these various aspects of thyroid function so as to define in what ways they may be interfered with. Substances which in one way or another depress the function of the thyroid gland may be classified under four headings: (1) Thyroid hormone; (2) Iodine; (3) Thiocyanate ion; (4) Antithyroid substances proper, compounds which interfere with thyroid hormone synthesis.

It is apparent that the structure and function of the thyroid gland are under delicate regulatory influences which insure that the quantity of hormone secreted is appropriate for the requirements of the body. We assume that, when thyroid hormone requirement is increased or when any influence tends to decrease the efficiency of thyroid hormone synthesis, there is a compensatory overproduction of thyrotropin from the pituitary, and the thyroid gland undergoes changes which we interpret as those of overactivity. Contrariwise, if the thyroid hormone requirement is reduced, or if extra thyroid hormone is artificially administered, there is a compensatory atrophy of the thyroid gland. This simple regulatory mechanism has been thought by many investigators to be inadequate to explain all of the phenomena which have been experimentally revealed. A number of investigators have suggested that there are stimulatory or inhibitory influences which act directly upon the thyroid gland and independently of the hypophysis. These direct influences include a direct stimulation of the thyroid gland by sympathetic nerve impulses; a direct inhibition of the thyroid gland by thyroid hormone or by iodine; a direct stimulus by low environmental temperature; etc. Other possible



regulatory influences outside of the simple scheme outlined above include the postulate of two hormones from the thyroid gland, only one of which suppresses thyrotropin production; the theory that there is an inhibitory hormone secreted by the pituitary gland; the claim that the thyroid may inactivate its own hormone; and the theory that, under certain circumstances, the thyroid gland inactivates thyrotropic hormone, or that this hormone is inactivated by iodine and potentiated by certain antithyroid substances. Some of these possible regulatory mechanisms will have to be considered in examining the mechanism of action of substances which inhibit thyroid function. If thyroid activity were regulated solely by the simple thyrotropin-thyroid hormone interplay, one would expect that, in the hypophysectomized animal, the thyroid gland could not be influenced by any means other than the administration of thyrotropin. One would also expect that thyroid hormone itself would have no action in either stimulating or depressing the various activities of the thyroid gland in the absence of the hypophysis. Without the hypophysis, also, there should be no structural change in the thyroid gland induced by changes in iodine intake, environmental temperature, nervous influences or antithyroid agents. Experiments have been reported, however, to show that in each of these instances positive effects could be obtained under certain circumstances in the absence of the hypophysis. When referring to thyroid activity, we must be careful to distinguish between the function of hormone synthesis and the activity as judged by histological criteria. A gland may appear cytologically to be in a resting or relatively inactive state and may yet be making a normal quantity of thyroid hormone. On the other hand, hormone synthesis may be completely blocked by an antithyroid agent while the gland appears to be very active indeed upon microscopic examination.

### *Thyroid Hormone*

The possibility that thyroid hormone may affect the thyroid gland directly has appealed to several investigators. There has been no substantiation of the claim (Pall, 1933) that thyroid hormone directly stimulates the oxygen consumption of thyroid tissue as it does that of certain other tissues; indeed, it has been claimed that there is an inhibitory action upon the respiration of thyroid tissue slices *in vitro* (Galli-Manini, 1941). Of course, the administration of thyroid to the intact animal results in regressive changes in the thyroid gland. This effect is identical with that induced by hypophysectomy, and it is tempting to conclude that the effect of thyroid is solely that of suppressing the thyrotropic activity of the hypophysis. However, the repeated demonstration, since Aron's (1930), that the concurrent administration of thyroid hormone with thyrotropin diminishes the thyrotropic effect of the latter is more difficult to explain. One could imagine that, in the intact animal, vigorous thyroid stimulation could exhaust the iodine supplied in the diet

and render the animal iodine-deficient. The animal's own pituitary might then be stimulated through a lowered production of thyroid hormone from the already activated thyroid. Under such circumstances, either iodine or thyroid hormone would appear to be inhibitory to the injected thyrotropin. Further evidence for a direct effect of thyroxine in inhibiting the action of thyrotropin upon the thyroid gland is provided by the work of Van Eck (1939), and of Cortell and Rawson (1944), who have shown that, in hypophysectomized rats, thyroxine decreases the effect of injected thyrotropin.

### *Iodine*

The several actions of iodine upon the structure and function of the thyroid gland are probably most complex, and the extensive studies of these iodine effects leave us with many unanswered questions.

It is clear that a deficiency in iodine intake and a consequent deficiency in thyroid hormone synthesis leads to the compensatory change in the thyroid gland which we recognize as hyperplastic goiter. The administration of iodine under such circumstances corrects the deficiency, permits thyroid hormone to be formed, and allows the thyroid gland to assume a more normal structure. In a sense, the administration of iodine under these circumstances evokes an antithyroid effect, that is to say, a marked degree of thyroid atrophy is induced, but of course thyroid hormone synthesis is promoted.

Two other actions of iodine upon the thyroid gland seem to be the direct antitheses of one another. The administration of iodine to persons suffering from hyperthyroidism results in a regressive change in the thyroid gland and in a diminished output of thyroid hormone. The exactly opposite situation obtains as regards thyroid hormone production when iodine is given to experimental animals or human beings with hyperplastic, non-toxic goiters. Marine and Lenhart (1909) showed that the administration of iodine to goitrous dogs had an action similar to the administration of thyroid hormone, *viz.*, the animals exhibited increased appetite, diarrhea, weakness, and weight loss for the first week or two but then usually recovered. Webster and Chesney (1928), on the other hand, found that iodine administered to goitrous rabbits induced severe hyperthyroidism which nearly always progressed to the death of the animal. These two variants of induced hyperthyroidism are also to be observed when iodine is given to human beings in goitrous districts. It would appear that the human counterpart more nearly resembles the condition seen in rabbits than that observed in dogs; that is, a self-perpetuating hyperthyroidism may be induced by the administration of iodine.

The striking effect of iodine in suppressing the thyroid and its function in hyperthyroidism remains completely unexplained. Marine and Lenhart (1911) likened this effect to that seen when hyperplastic en-

demic goiters were treated with iodine. They supposed that the iodine permitted the thyroid to make its normal hormone instead of a perverted one. Various mechanical theories have been proposed around the general idea that the thyroid becomes packed full of hormone when presented with such large amounts of building material and that the pressure of the accumulated secretion then shuts off the blood supply and prevents the leaching out of hormone into the rest of the body. More appealing are the theories which attempt to define more precisely just how this phenomenon is brought about. Loeser and Thompson (1934) found that, when iodine was administered to the normal animal, the quantity of thyrotropin hormone in the pituitary was reduced. This would be in keeping with the explanation that iodine shuts off the supply of thyrotropic hormone and such an effect, of course, would explain the atrophic change in the thyroid and the decreased production of thyroid hormone. However, this effect of iodine might be mediated by the thyroid hormone formed from it and not by an action of the iodine itself upon the pituitary.

The theory of a direct inhibitory action of iodine upon the thyroid gland also has substantial documentary support. The earlier experiments showing that iodine would prevent the compensatory hyperplasia which followed partial thyroid ablation were qualified by the work of Marine and Lenhart. They showed that the administration of iodine would not prevent the regrowth of thyroid tissue after partial thyroidectomy in dogs that possessed normal-appearing thyroid glands. If, however, the experiment were performed on dogs with hyperplastic, presumably iodine-deficient thyroids, then the iodine caused a reversion of the hyperplastic glands to normal ones even though large portions of the goiters had been removed surgically. They concluded that iodine would prevent hyperplasia but not compensatory regrowth of thyroid tissue. A very different concept of the action of iodine on the thyroid gland was put forth by Loeb (1920). Extensive studies by Loeb and collaborators showed that not only was compensatory hyperplasia not prevented by iodine, but iodine increased the degree of hyperplasia following partial thyroidectomy. They showed further that the administration of iodine alone to normal guinea pigs would be followed by a wave of mitotic activity associated with changes in the colloid which the authors took to indicate a stimulating action of iodine upon the thyroid gland. This effect of iodine has received little further attention and remains entirely unexplained. Later, however, Silberberg (1929) and Siebert and Thurston (1932), from Loeb's laboratory, demonstrated that the administration of potassium iodide to normal guinea pigs would inhibit partially the stimulating action of thyrotropic hormone upon the thyroid gland and, to some degree, the increased metabolism induced by thyrotropin. These results have been confirmed by Friedgood (1935), Anderson and Evans (1937), Cutting and Robson (1939), Trikojus (1939), and others. Further evidence in support of this theory was provided by Vanderlaan,

Vanderlaan, and Logan (1941), who showed that the respiratory metabolism of thyroid slices from guinea pigs treated with iodide as well as thyrotropic hormone was less than in other animals given thyrotropic hormone alone. Loeser and Thompson (1934) observed no effect of iodine in inhibiting thyrotropin in the absence of the hypophysis. Another line of evidence supporting the theory that iodine inhibits the action of thyrotropic hormone upon the thyroid gland is provided by experiments performed with antithyroid agents such as thiouracil. McGinty and Sharp (1946) have shown that, if the iodide intake is increased, there is a slight but distinct inhibition of the thyroid enlargement induced by thiouracil. This observation has been amply confirmed, and one theory which could explain the phenomenon is that the accumulated iodide either suppressed the thyroid gland directly or inhibited the action of thyrotropin upon it. There is good reason to believe that the added iodine does not act by permitting thyroid hormone to be formed. Wright and Trikojus (1946) have carried the theory one step further and have proposed that it is not the iodide which inhibits the thyrotropin, but that within the thyroid gland iodide is converted to iodine and this reacts with the thyrotropin to inactivate it. Rawson and co-workers (1946) have made extensive studies on the inactivation of thyrotropin by iodine and its reactivation by various reducing agents, including antithyroid compounds. Any theory of the mechanism of action of iodine must take into consideration the fact that added iodine in the normal animal has no detectible effect upon the thyroid gland.

Of course, one might ask: "What is a normal thyroid gland?" In order for it to be normal, there must be available an adequate iodine intake, but where is one to draw the line between an inadequate and an adequate supply of iodine? The work of Levine, Remington, and von Kolnitz (1933) showed that, as the iodine in the diet is increased from deficiency levels upward, there is a gradual decrease in the resulting size of the thyroid gland and a gradual increase in the amount of iodine stored therein. At no point can one draw the line and say where deficiency stops and excessive iodine intake begins (FIGURE 1). Similarly, Taurog and Chuikoff (1946) have shown that there is a progressive increase in the total of thyroxin iodine in the thyroid and in the protein-bound iodine of the plasma of rats as the daily iodine intake is increased up to as much as 78 micrograms per day (FIGURE 2). One might argue that, if the dietary iodine is already adequate or excessive, the addition of more iodine will then have no demonstrable effect upon the normal thyroid gland. Perhaps a more satisfying theory would not hold that iodine suppresses the thyroid gland directly or inhibits the action thereon of thyrotropic hormone, but one might state the proposition that a deficiency of iodine within the thyroid cell is a stimulus to it. The most direct evidence in support of this possibility is that of Chapman (1941), who observed an increase in the size, vascularity, and hyperplasia of the thyroids of hypophysectomized rats fed a low iodine diet.

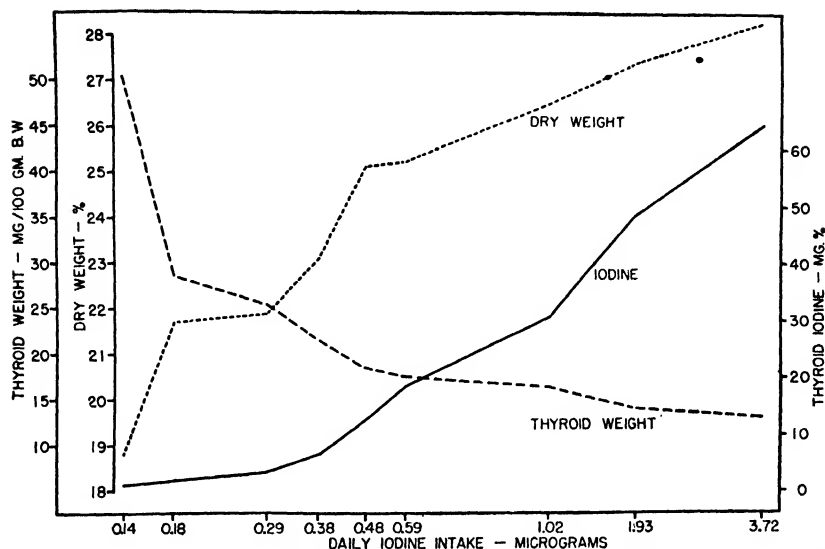


FIGURE 1. The effect of the prolonged feeding of diets of varying iodine content upon the resulting weight, dry weight, and iodine concentration of the thyroid glands of rats. The thyroid weight is expressed in milligrams per 100 grams of body weight, and the iodine concentration is expressed in milligrams per 100 grams of fresh thyroid tissue. The iodine intake in micrograms per day is plotted on a logarithmic scale. (Redrawn from the data of LEVINE, REMINGTON, & VON KOLNITZ, 1933.)

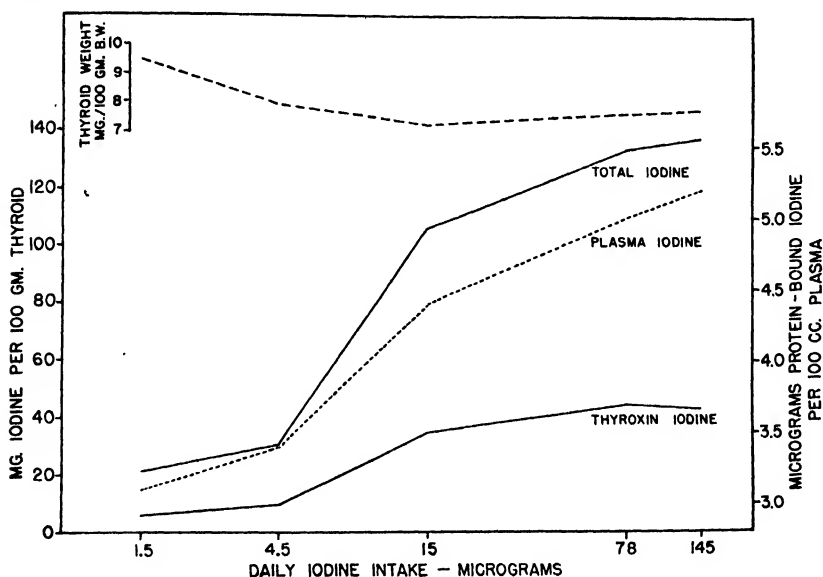


FIGURE 2. The effect of various quantities of dietary iodine upon the thyroid weight, the plasma protein-bound iodine, the total, and thyroxine iodine concentrations in the thyroid glands of rats. The thyroid weight is expressed in milligrams per 100 grams of body weight, the total and thyroxine iodine concentrations are in milligrams per 100 grams of wet thyroid, and the protein-bound iodine is in micrograms per 100 cubic centimeters of plasma. The iodine intake in micrograms per day is plotted on a logarithmic scale. (Redrawn from the data of TAUBO & CHAIKOFF, 1946.)

It would seem likely that a proper understanding of the action of iodine on the thyroid gland of experimental animals would lead to a solution of the puzzling question of how iodine benefits patients with Graves' disease. De Robertis and Nowinski (1946) have shown that, if material from the thyroid gland containing the proteolytic enzyme, which they regard as important for the mobilization and secretion of thyroglobulin, is treated with iodine, the proteolytic activity is destroyed. He proposes this as the explanation of the action of iodine in Graves' disease. It might be well to recall that the treatment of a protein enzyme with iodine is a vigorous chemical procedure, and such treatment is likely to inactivate most enzyme systems. It is doubtful that such a reaction would take place within the thyroid gland. Morton, Chaikoff, and Rosenfeld (1944) have shown that the synthesis of thyroid hormone by thyroid tissue slices *in vitro* is inhibited by large excesses of iodide ion in the medium.

The extensive work on iodine and the mechanism of its action in Graves' disease leaves us without an entirely satisfactory explanation.

Let us examine, then, the theory that a decreased quantity of iodide ion in the thyroid cell is a direct stimulus of that cell's activity. According to this theory, one should find that goiter induced by iodine deficiency should be far more striking than that induced by such antithyroid agents as thiouracil, for not only would there be compensatory hyperplasia induced by overproduction of the thyrotropin, but the gland would, according to theory, be stimulated directly by the low iodide concentration within it. The largest goiters experimentally induced are probably those of Chesney, Clawson, and Webster (1928) in rabbits fed on cabbage. If, as seems likely, the diet not only provided a goitrogen but was at the same time iodine-deficient, the findings would be in keeping with the theory. The effect of iodide on the goiter induced by antithyroid agents, such as thiouracil, is ever so much more striking in the range of partial iodine deficiency than with very large quantities of added iodine. That is, an iodine-deficient diet induces a great potentiation of goitrogenic effect, whereas iodine added to a "normal" diet only slightly inhibits the goiter. Considering, then, the effects of iodide on Graves' disease, one might suppose that in that condition an individual on a normal diet is indeed iodine-deficient. The turnover of iodine and thyroid hormone is ever so much increased, and, as shown by Curtis and Puppel (1938), hyperthyroid individuals are in negative iodine balance when eating a normal diet. Thus, one might suppose that the iodide concentration in the thyroid cell of Graves' disease is very low. This may be one link in the chain of events which leads to the self-perpetuation of hyperthyroidism in man. Iodide administration under these circumstances would restore the normal iodide concentration in the thyroid and cause a certain degree of thyroid atrophy by withdrawing the stimulus of a low iodide concentration. It must be emphasized that this is pure speculation.

### *Thiocyanate*

Recent work demonstrates that the thiocyanate ion exerts a unique effect upon the thyroid gland, shared by no other substance yet known. The first indications of the effect of thiocyanate ion upon the thyroid gland were provided by the observations of Barker (1936) on patients with hypertension, treated with potassium thiocyanate. It was observed that, among other toxic effects, so called, a certain number of patients experienced enlargement of the thyroid gland. The patients exhibited a myxedema-like swelling of the tissues of the face, and the administration of 1 to 2 grains of thyroid daily reversed the changes within two weeks (Barker, Lindberg, and Wald, 1941). Similar observations were subsequently made by others, and in 1942 it was shown by Kobaker (1942) and by Rawson, Hertz, and Means (1942, 1943) not only that goiter may result from thiocyanate administration, but that the goiter may be associated with all the symptoms and signs of myxedema. Thiocyanate was also found to be goitrogenic in animals, but it was soon apparent that it differed from the large groups of antithyroid compounds in being readily inhibited by increasing the iodine intake (Astwood, 1943). Even the quantities of iodine contained in commercial rat food were nearly adequate to prevent the goitrogenic effect of thiocyanate given for short periods of time. Thiocyanate was also set apart from other goitrogens by the findings of Franklin, Chaikoff, and Lerner (1944), showing that thiocyanate inhibits the uptake of iodine by surviving thyroid slices *in vitro*, whereas other antithyroid agents have little effect at concentrations which effectively prevent hormone synthesis.

An interpretation of the extensive studies on iodine metabolism in the thyroid gland and of the inhibitory actions of antithyroid compounds requires that a distinction be made between *iodide* uptake by the thyroid gland and the total *iodine* accumulation. One would expect that, if there were a large amount of inorganic iodine in the thyroid gland, the administration of tracer doses of radioactive iodine would result in a very large and rapid accumulation of radioactivity in the thyroid gland. This is not the case, as the experiments of Perlman, Chaikoff, and Morton, as well as of Leblond (1942), show. Rather, there is a steady increase in radioactivity in the gland for some hours after the administration of a tracer dose (FIGURE 3). This accumulated iodide is largely protein-bound. Lein (1943) fractionated the thyroid iodine of rabbits into acetone-soluble (inorganic) and acetone-insoluble (protein-bound) iodine following the injection of 35 micrograms of iodine labeled with  $I^{131}$ . As shown in FIGURE 4, the major portion of the radioactivity accumulated slowly in the protein fraction. At the end of twelve hours, the quantity in this fraction amounted to about 1/40 of the injected dose and was still increasing. The inorganic fraction contained very little radioactivity at any time, but the maximum of about 1/200 of the injected dose was ac-

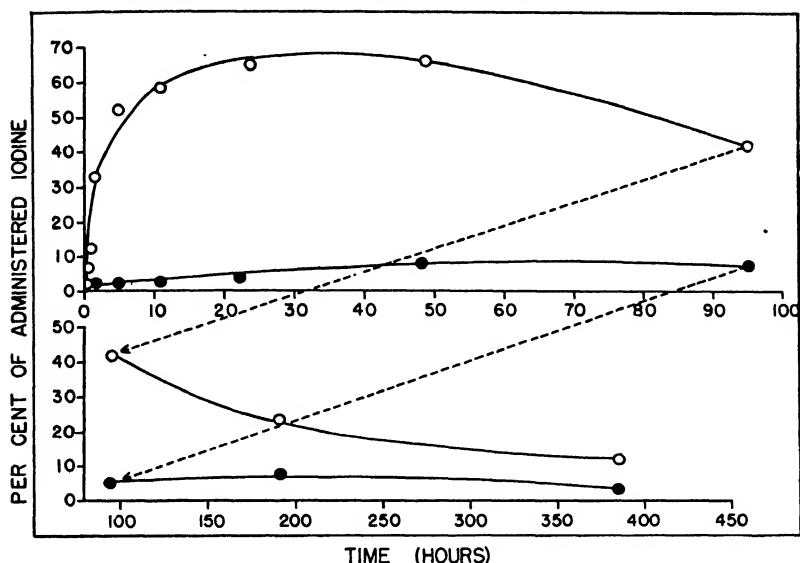


FIGURE 3. The uptake of labeled iodine by the thyroid gland of the rat. Open circles, each animal receiving a tracer dose of labeled iodine; solid dots, each animal receiving 0.03 mg. of labeled iodine. Each point represents the average of four separate analyses on as many animals. (From PERLMAN, CHAIKOFF, & MORTON, 1941.)

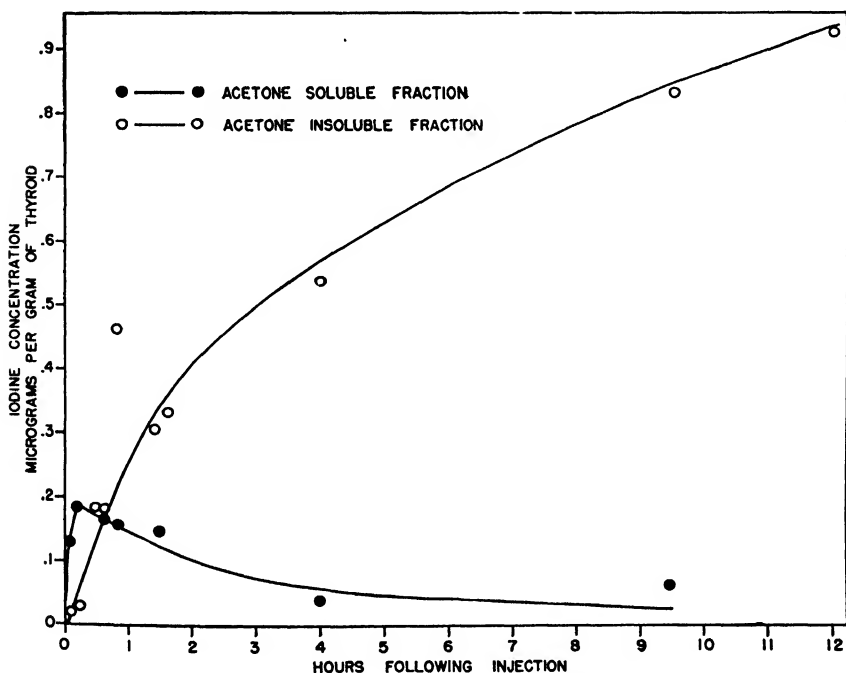


FIGURE 4. Concentration of acetone-soluble and acetone-insoluble labeled iodine in the rabbit thyroid after the intravenous administration of 35 mg. of soluble labeled iodide. (From LEIN, 1943.)



cumulated within a few minutes. The data of Morton, Perlman, Anderson, and Chaikoff (1942), and of Leblond and co-workers (Leblond and Sue, 1941; Mann, Leblond, and Warren, 1942; Leblond, Puppel, Riley, Radike, and Curtis, 1946), also indicates that the major portion of injected radioactive iodine when given in tracer doses appears relatively slowly in a protein-bound form (FIGURE 5). These experiments provide

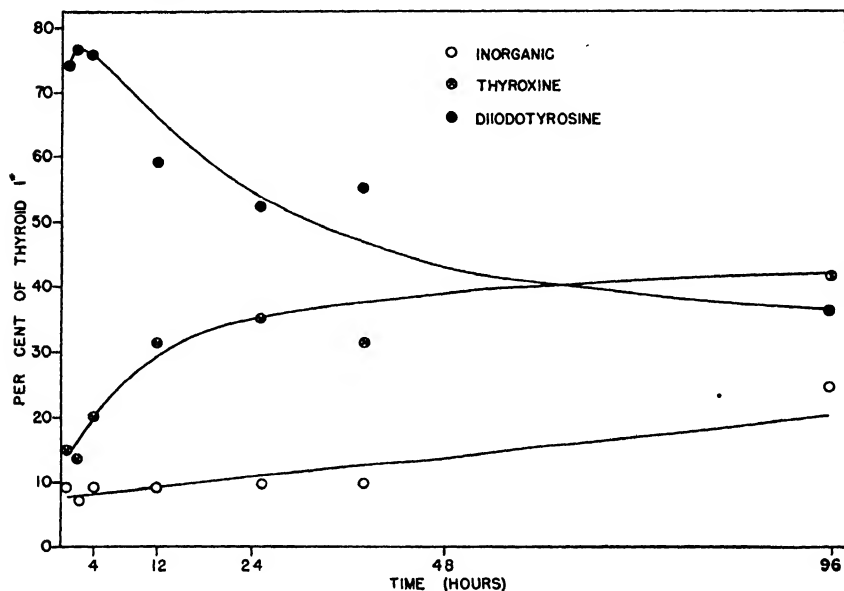


FIGURE 5. Distribution of thyroid radioactive iodine among inorganic, diiodotyrosine, and thyroxine fractions in normal rat. (From MORTON, PERLMAN, ANDERSON, & CHAIKOFF, 1942.)

further evidence for the belief that very little inorganic iodide is held in the normal thyroid gland (Harrington, 1944). This situation contrasts sharply with that of thyroid glands depleted of their normal stores of hormone by thiouracil. If normal rats are treated with thiouracil for about ten days, the total iodine in the thyroid gland is markedly reduced, and if a dose of iodine is then given in the form of iodide, there is a very rapid uptake of iodine by the thyroid gland, reaching a maximum in fifteen to sixty minutes (Astwood, 1945; Vanderlaan and Bissell, 1946a). This markedly concentrated iodine behaves as though it were still iodide ion. Vanderlaan and Vanderlaan (1947) have shown that it is not precipitated by protein precipitants, that it readily filters through cellophane membranes, and in the ultrafiltrate both amperometric and potentiometric titrations show that it behaves in all respects like iodide ion. The magnitude of the concentration of iodide ion under these circumstances is truly remarkable. Taking an isolated case where 100 micrograms of potassium iodide is administered to 100-gram rats, assuming that this iodide is then distributed into 30 per cent of the body weight, one would have a maximal concentration of iodide in the extra

cellular fluid of 0.257 mg.%, whereas in the thyroid gland the concentration is 25 mg.% as a minimal figure. This represents a hundred-fold concentration. Vanderlaan and Vanderlaan have actually determined the ratio of thyroid-blood iodide after various doses of iodide, using  $I^{131}$  to label each administered dose. Following doses of 100 micrograms or less of potassium iodide, the hyperplastic thyroids of propylthiouracil-treated rats contained about 250 times as much iodide as did blood serum. The resting glands of untreated animals contained twenty-five times the amount to be found in an equal volume of blood serum (FIGURE 6). These findings show that compounds such as thiouracil do not inter-

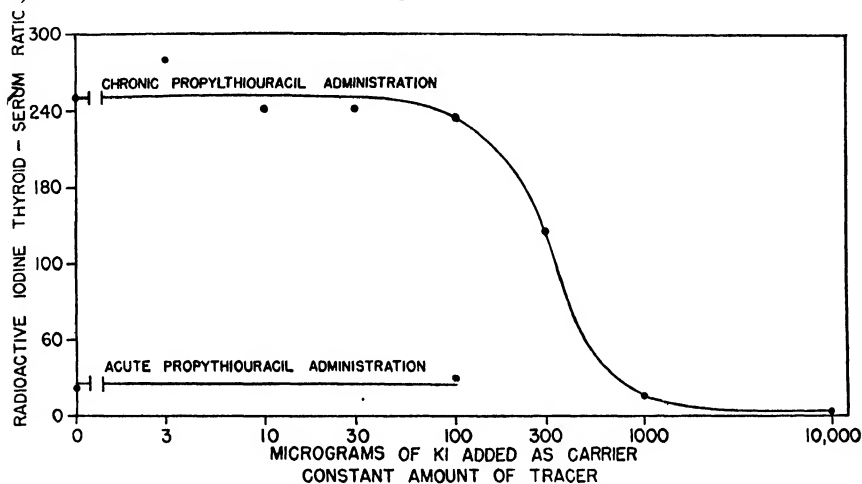


FIGURE 6. Ratio of the concentrations of radioactive iodine in the thyroid gland and serum one hour following the injection of various doses of potassium iodide labeled with radioactive iodine. The values shown in the upper curve were obtained in animals pretreated for two weeks with propylthiouracil in the diet. Those in the lower curve were obtained in previously untreated animals given a single dose of propylthiouracil before the injection of the iodine. The large, iodine-depleted glands of propylthiouracil-treated animals concentrated iodide some 250 times the serum concentration except when doses greater than 100 micrograms were given. In the normal thyroids, the iodide concentration was only about 25 times the serum concentration. (From VANDERLAAN & VANDERLAAN, 1947.)

fere with this step of thyroid function, but they indicate that the thyroid gland, when depleted of hormone, has an enormously expanded capacity for iodide. Wolff, Chaikoff, Taurog, and Rubin (1946), as well as Vanderlaan and Bissell (1946), have shown that thiocyanate markedly inhibits the accumulation of iodide by the thyroid gland. Vanderlaan and Vanderlaan (1947) have further shown that, when as little as 1 mg. of potassium thiocyanate is administered to animals whose thyroid glands contain a large quantity of inorganic iodide, there is an immediate discharge of all of it from the thyroid gland (FIGURE 7). This phenomenon can also be demonstrated in the human being through the use of radioactive iodine (FIGURE 8). This extraordinary effect at first suggested the possibility that thiocyanate was selectively absorbed by the thyroid gland, thus displacing iodine ion. It is known that thiocyanate may be regarded as a halide, since it behaves as such in many chemical reac-

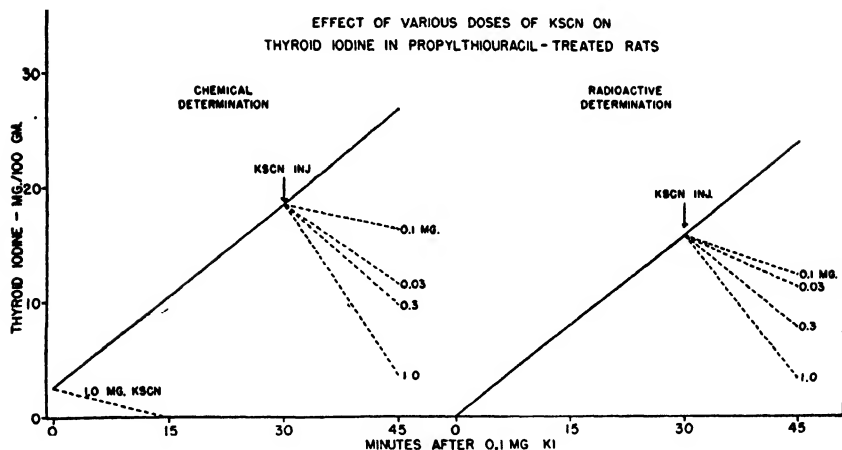


FIGURE 7. The effects of various doses of potassium thiocyanate upon the concentration of iodine in the thyroid gland of rats pretreated with propylthiouracil and injected with 100 micrograms of potassium iodide. The solid lines show the rise in thyroid iodine following the injection of iodide, and the dashed lines indicate the fall following the injection of thiocyanate. The analyses were made by chemical methods and by the use of radioactive iodine as a tracer. (From VANDERLAAN & VANDERLAAN, 1947.)

tions. It is also known that it is selectively excreted by the salivary glands, that it interferes with the excretion of hydrochloric acid in the stomach (Davenport, 1940), retards the absorption of chloride ion from the intestinal tract (Driver, 1942), and so forth. However, in no place in the body is there any appreciable concentration of thiocyanate (Corper, 1915), and it is distributed largely in the extracellular fluid. Actual measurements show that under the above circumstances there is no accumulation of thiocyanate in the thyroid gland, the blood level always remaining higher than the thyroid tissue level. The mechanism, then, whereby thiocyanate renders the thyroid incapable of concentrating iodide ion remains to be explained. The effect is so striking that it raises again the question of how important thiocyanate might be to thyroid function under normal circumstances.

The thiocyanate ion is widely distributed. It occurs in the blood and, particularly, in the saliva of normal persons and is excreted in the urine; presumably, it represents the detoxication product of cyanide. It occurs in appreciable concentrations in many plants, such as members of the *Brassica* family and Umbelliferae. Perhaps even more important than the occurrence of free thiocyanate in plants is the occurrence of substances which are turned into thiocyanate by the mammalian organism. These include the isothiocyanates, or mustard oils, the organic nitriles, and the widely distributed cyanogenetic glucosides. It seems quite possible that the consumption of foods containing these substances would render iodine-deficient a diet which otherwise would be adequate in iodine, and might explain the fact that endemic goiter exists in regions of the world where the iodine of the soil and water is abundant.

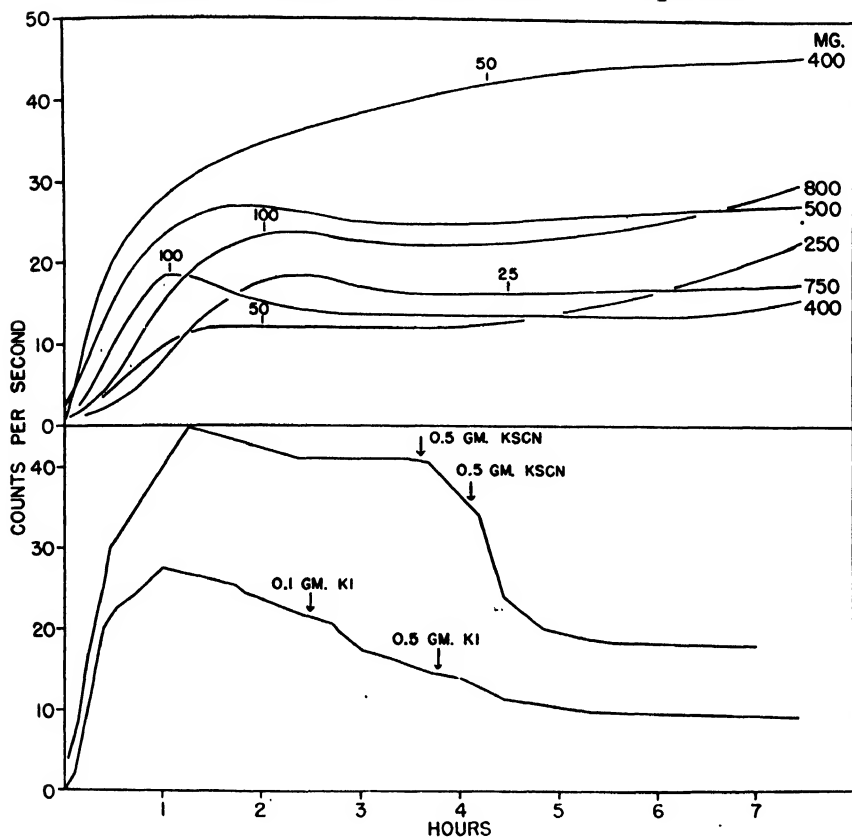


FIGURE 8. The upper part of the figure illustrates the effect of pretreatment with propylthiouracil on the accumulation of radioactive iodine in the thyroid region of normal human subjects. In each case, the first dose of propylthiouracil was given one to two hours before the 0.1 millicurie dose of radioactive iodine. The size of the last dose is shown on each curve at the time of its administration, and the total doses of propylthiouracil are given at the right side of the figure. It may be noted that four of the six subjects exhibited a fall in concentration after the maximum had been reached. The values for five of the six subjects began to rise again as the effect of propylthiouracil wore off. The curves in the lower half of the figure illustrate the effect of potassium thiocyanate and of potassium iodide on the concentrating radioactive iodine in the thyroid region in subjects pretreated with propylthiouracil. The iodine concentrated in the thyroid region presumably remains in the form of iodide which is freely diffusible. Potassium thiocyanate inhibits the iodide-entrapping mechanism, and the large doses of ordinary iodide dilute out the accumulated radioactive iodide. (From Astwood & Stanley, 1947.)

The earlier finding that thiocyanate is not goitrogenic when iodide is added to the diet might have the following explanation: The remarkable capacity of the hyperplastic thyroid gland to concentrate iodide might be of the greatest importance when the dietary iodine is low; without this special mechanism, or if it were inhibited by thiocyanate, hormone synthesis would be impeded owing to the very dilute iodide substrate. With added iodine in the diet, iodide in adequate quantities might reach the thyroid gland by simple diffusion and thus hormone synthesis could proceed at a normal rate even in the presence of thiocyanate.

*Antithyroid Substances*

There are a great many compounds which fall into this category. They can be divided roughly into two main groups, the most active of which are those which have in common a thiocarbonamide grouping typified by thiourea and related compounds, and secondly those with an amino-benzene grouping, the best known of which are the sulfonamides, and the most active such compounds as 4,4'-diaminodiphenylmethane and 4,4'-diaminobenzil. It is now generally agreed that these two groups of substances act by inhibiting the synthesis of thyroid hormone but have no action upon the iodide-accumulating mechanism and no direct effect upon the thyroid gland itself. A consideration of their mechanism of action is intimately related to the poorly understood process of thyroid hormone synthesis—the conversion of iodide into an organic form. This step presumably involves an oxidation wherein an electron is removed from iodide ion, permitting it to take the form of iodine or to be immediately incorporated into tyrosine. Now, it is abhorrent to current biochemical thought that free iodine should for any instant exist in the thyroid cell, nor can it be imagined that this free iodine could react with water to form hypoiodite and iodide or that hypoiodite could exist for any appreciable time. Nonetheless, iodide ion somehow becomes incorporated into tyrosine, and whether or not iodine or hypoiodite is ever free is a less important consideration than the fact that iodide must be oxidized. Such an oxidation could, of course, be carried out by oxygen itself, by free hydrogen peroxide, or by hydrogen peroxide catalyzed by peroxidase. The other commonly recognized enzymes of tissue presumably could not carry out this oxidation, owing to the high potential involved.

A study of the well-known reaction of thiourea-like substances with iodine has shown that, at neutral pH, free iodine is reduced by such substances as thiouracil (FIGURE 9). The rate of this reaction is so rapid that

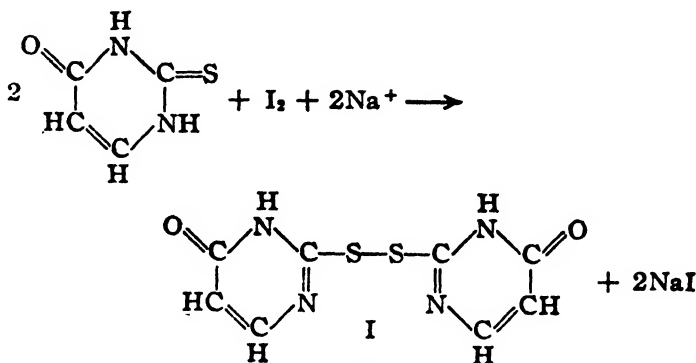


FIGURE 9.

it cannot be accurately measured *in vitro*, but it is likely that it is many hundred times the rate of the reaction between iodine and tyrosine. The view put forth by Miller, Roblin, and Astwood (1945) that this reaction

might explain the antithyroid effect has numerous points in its favor. Each of the many thiourea-like compounds known to affect the thyroid gland reacts rapidly with iodine. There are, however, many substances such as cystine, glutathione, and thioglycollic acid, which react with iodine at a similar rate, but which do not interfere with thyroid function. It is necessary, then, to postulate that these inactive substances either do not reach the appropriate site in the thyroid cell, or else they do not reach there in a reduced form. It is interesting to speculate that the iodide-to-iodine oxidation possesses a redox potential much higher than other common oxidations in the body, and perhaps the active antithyroid agents must possess a redox potential in this range in order to be effective, having survived other common oxidative processes.

In considering the aminobenzene derivatives, it is immediately apparent that this explanation of antithyroid action cannot be applied. They react only very slowly with iodine, and could not be regarded as competitive with the iodination mechanism. Physiological experiments, however, have not yet established any clear difference between the action of the thiocarbonamide substances and the aminobenzene group, with the possible exception of the work of MacKenzie and MacKenzie (1943) and MacKenzie (1947), who showed that under certain circumstances the goitrogenic effect of sulfaguanidine is actually potentiated slightly by added iodine. Most other workers agree that increasing the iodide concentration in the diet diminishes slightly the goitrogenic effect of thiouracil and related substances. Whether this observation will lead to a distinction as to the exact mode of action between the two groups of substances cannot yet be stated.

The second step in the synthesis of thyroid hormone presumably involves another oxidative process permitting the formation of the diphenyl ether between two diiodotyrosine residues with the loss of one alanine side chain. The findings of Morton, Perlman, Anderson, and Chaikoff (1942) indicate that the hypophysis is essential to the process (FIGURE 10—compare with FIGURE 5). This oxidation is one which can also be promoted by such oxidants as iodine, hydrogen peroxide, and atmospheric oxygen, and, as Westerfeld and Lowe (1942) have shown, it can be catalyzed by peroxidase in the presence of hydrogen peroxide. It is immediately apparent that this oxidation might be very similar, if not identical, to the preceding one, and agencies which inhibit one might be supposed to inhibit the other. One could equally well reason, however, that biological oxidation can be most specific and that aminobenzene derivatives could exert their antithyroid effect by a competitive mechanism in this step due to chemical similarity between the aminobenzene radicals and diiodotyrosine (Astwood, 1943). Support for this hypothesis was thought to be provided by the fact that the administration of relatively large amounts of diiodotyrosine did not inhibit the goitrogenic effect of the sulfonamides, indicating that, though diiodotyrosine was available, this could not be converted to thyroid hormone

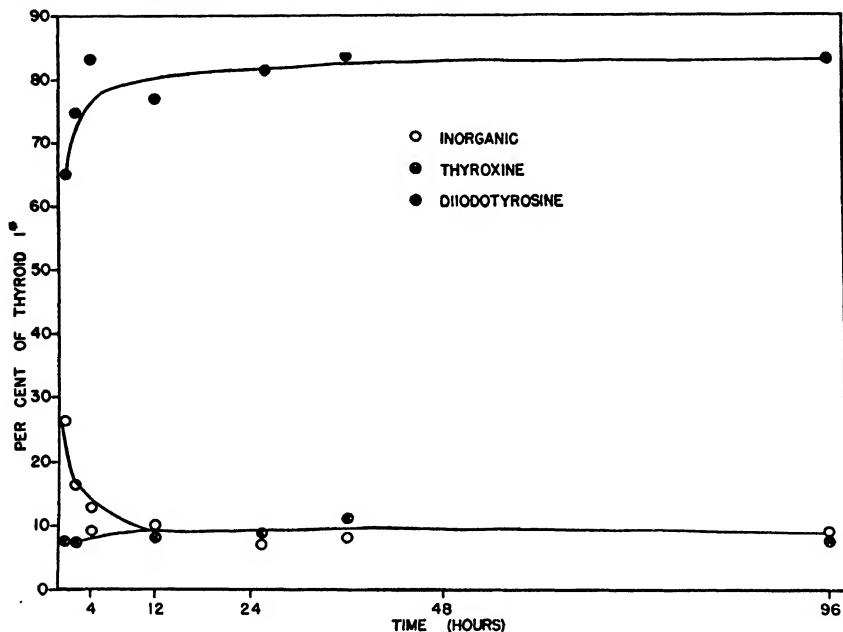


FIGURE 10. Distribution of thyroid radioactive iodine among inorganic, diiodotyrosine, and thyroxine fractions of the hypophysectomized rat. (From MORTON, PERLMAN, ANDERSON, & CHAIKOFF, 1942; compare with FIGURE 5.)

in the presence of the goitrogen. Further consideration, however, shows that this line of reasoning is fallacious, because it is highly probable that artificially administered diiodotyrosine is not utilized by the thyroid gland but is broken down in the body and only the resulting iodide ion is available to the thyroid gland (Snapper, 1938). Harrington (1944) has recently suggested that thyroxine itself is the hormone of the thyroid gland and that its synthesis goes on from the free amino acid tyrosine. When regarded from different points of view, this theory does not seem to be the correct explanation. The *in vitro* conversion of diiodotyrosine to thyroxine is a very slow process, and no matter what conditions are employed, the yields are minute (Harrington, 1934; Reineke and Turner, 1946). On the other hand, the data of Reineke and Turner (1943), showing that perfectly enormous yields of thyroxine can result from the proper management of iodinated casein, suggests that within the thyroid gland thyroid hormone synthesis goes on at the level of protein molecules rather than as amino acids. If this be the case, then artificially administered diiodotyrosine would not enter into the synthesis of thyroid hormone. We have no evidence, then, that any antithyroid substance interferes with the conversion of diiodotyrosine residues to thyroxine residues within this thyroid protein. The extensive work with radio-iodine likewise fails to reveal any inhibition at this step.

*Specific enzyme inhibition* is an attractive theory of the action of anti-

thyroid compounds, and several enzymes have been suggested as important to thyroxine synthesis. The *cytochrome-cytochrome oxidase* system was shown by Schackner, Franklin, and Chaikoff (1943) to be essential to the synthesis of thyroid hormone *in vitro* by thyroid tissue slices. When this system was poisoned by azide, sulfide, or cyanide, thyroxine was not formed. However, these compounds depressed thyroid tissue respiration, whereas effective antithyroid agents did not (Lerner and Chaikoff, 1945). Morton and Chaikoff had previously presented evidence to show that intact surviving thyroid tissue was essential to thyroid hormone synthesis *in vitro*, and that homogenized tissue was ineffective. Perhaps, then, poisoning of the cytochrome system is a non-specific effect and merely serves to kill the cells the vitality of which is essential to the synthetic process. The claim that cytochrome oxidase is inhibited by thiouracil (Paschkis, Cantarow, and Tillson, 1945) has been denied by Glock (1946), who has presented evidence to show that thyroxine synthesis is not directly concerned with cytochrome oxidase. DuBois and Erway (1946), McShan, Meyer, and Johansson (1946), and Tipton and Nixon (1946) likewise found no inhibitory action of thiourea derivatives and sulfonamides on cytochrome oxidase or succinic dehydrogenase. In view of the highly specific nature of the antithyroid effect, it would be unlikely that it could be mediated by an inhibition of such a widely distributed, ubiquitously important enzyme system as cytochrome-cytochrome oxidase.

*Tyrosinase* is another enzyme which is inhibited by certain antithyroid compounds. These compounds include phenylthiourea, under certain circumstances para-aminobenzoic acid and sulfanilamide, and thiouracil and other thiourea derivatives (Bernheim and Bernheim, 1942; Martin, Wisanski, and Ansbacher, 1941; Paschkis, Cantarow, Hart, and Rakoff, 1944). Roberts (1945) has studied the inhibitory action of various antithyroid compounds on tyrosinase, using as substrates *L*-tyrosine, *L*-dihydroxyphenylalanine, *p*-cresol, and homocatechol. Some correlation was found between antithyroid activity and antityrosinase action. The best correlation was observed when *L*-tyrosine was the substrate. Under these conditions, thiourea, methylthiourea, guanlythiourea, para-aminobenzoic acid, and sulfanilic acid were inhibitory, while methylisothiourea sulfate and the sulfonamides were not. DuBois and Erway (1946) studied several thiourea derivatives and found that antityrosinase action paralleled closely their toxicity for rats but not their antithyroid activity. This observation and the lack of evidence for the existence of tyrosinase in the thyroid gland suggests that the antithyroid activity of these agents is not mediated by an inhibitory action on this enzyme.

*Peroxidase* of the several enzymes which might be concerned in thyroxine synthesis seems the most likely. It is capable of promoting the oxidation of iodide and, as pointed out by Westerfeld and Lowe (1942), it could carry out the oxidative coupling to two diiodotyrosine molecules



to form thyroxine. Peroxidase is inhibited by sulfonamides (Lipman, 1941), by thiourea (Sumner and Somers, 1943), and thiouracil (Glock, 1944). Dempsey has described fine granules in the thyroid cell which give histochemical reactions typical of peroxidase and has shown that thiouracil inhibits these reactions. Glock (1944) has raised the objection that no peroxidase can be extracted from thyroid tissue. The difficulty in arriving at a decision as to whether a true peroxidase exists in the thyroid gland is caused by the numerous substances, chiefly iron porphyrin compounds, which have a peroxidase effect. Even if these substances are properly termed pseudo-peroxidases one of them could still be concerned in thyroid hormone synthesis. Dempsey's observations were confirmed by De Robertis and Grasso (1946), who found, however, that while thiourea inhibited the peroxidase reactions, sulfonamides did not. A most ingenious experiment was reported by Keston (1944). Radioactive iodide and xanthine were added to milk, and, after incubation, thyroxine containing radioactive iodine was isolated. Presumably, the xanthineoxidase of the milk acting upon the added xanthine liberated hydrogen peroxide, which in the presence of milk peroxidase oxidized the iodide to iodine, and this, in turn, iodinated the tyrosyl residues of the casein. The iodinated casein then formed thyroxine. No thyroxine was isolated if thiourea was added before incubation. This experiment may be a model of the normal mechanism of thyroxine synthesis. Randall (1946) has tested a series of 35 thioureas as well as sulfonamides and other compounds on the system  $\text{H}_2\text{O}_2$ : horseradish peroxidase: para-aminobenzoic acid. It was found that the thiol compounds were oxidized by peroxidase and thus competed with the substrate for the enzyme and the  $\text{H}_2\text{O}_2$ . If peroxidase is normally concerned in thyroid hormone synthesis, then substances such as thiouracil could interfere in several different ways. In the reaction iodide-iodine, thiouracil could: (1) reduce the iodine as fast as it is formed—in other words, inhibit the enzyme; (2) compete for the enzyme and be oxidized instead of iodide; (3) inhibit the enzyme; and (4) compete for the available  $\text{H}_2\text{O}_2$  so that none is available for the oxidation of iodide. It is conceivable, too, that the reaction, diiodotyrosine-thyroxine, is also mediated by peroxidase, in which case thiouracil could also inhibit by competing for the enzyme or the  $\text{H}_2\text{O}_2$  or inhibit the enzyme by some other means.

The action of aminobenzene derivatives is also difficult to explain. We must assume that they act to prevent the organic binding of iodine, but as they do not iodinate readily and are not effective in reducing iodine they cannot act directly on iodine. Like the thioureylenes, they could inhibit peroxidase or compete with the substrate or the  $\text{H}_2\text{O}_2$ , but it is also possible that through structural similarities with tyrosine they could inhibit iodination by competition for free iodine or hypoiodite. This could be considered a competitive reaction even though very little actual iodination takes place.

*Other possibilities.* The well-known property of thiols, of combining

with certain metals to form complexes, suggests the possibility that the synthesis of thyroxine is catalyzed by a metal or a metal-containing enzyme and that thiourea-like compounds act by containing with the metal. Reineke and Turner (1945) found that certain manganese compounds would increase the yield of thyroxine if present during the incubation of iodinated casein, and, recalling the finding that manganese increases the oxygen consumption of rats, suggested that manganese may be concerned in thyroid hormone synthesis. Considering the possibility that some unusual metal protein might be concerned with hormone synthesis, a search of the literature on tissue elements was made. No clear evidence of an unusual concentration of any element except iodine was found. Consequently, a spectrographic analysis was made of the ash of thyroid and liver of pigs. These were semiquantitative comparisons of the arc spectra made at the spectrochemical laboratory of the Massachusetts Institute of Technology. The first comparison showed a preponderance of chromium in the thyroid sample. When this was repeated with care to avoid contamination from metal instruments, no chromium was detected. As was to be expected, the liver samples contained more trace elements and larger quantities of them than the thyroid. No evidence for an unusual concentration of any element in the thyroid was found. While these findings are negative evidence that such elements as zinc, cobalt, copper, manganese, or magnesium are concerned with thyroxine synthesis, they are not against the proposition that an iron-containing enzyme is involved. The abundance of iron in tissue would obscure small differences in iron content.

Other antithyroid effects have been omitted from this review because of lack of information. Since the original description, by Moebius, of the therapeutic value of the serum of thyroidectomized animals, many agents have been claimed to have antithyroid properties. Besides various tissues, tissue extracts and plant materials, compounds such as diiodotyrosine, and bromine and fluorine derivatives, especially those of tyrosine, have been said to be beneficial in hyperthyroidism. The most recent of these was 3-fluoro-phenylacetic acid, which was introduced in Germany during the recent war. Animal experiments with this compound were recently carried out here with entirely negative results. It neither inhibited thyroid function nor interfered with the action of thyroid or thyroxine.

Woolley (1946) has recently claimed to have obtained a direct inhibition of thyroxine action by the use of various ethers of *N*-acetyldiiodotyrosine. Even with the most active compound, the paranitrophenylether, relatively very large quantities were required. The experiments would be more convincing had they been made with more specific test reactions. However, an effective thyroxine antagonist would be most useful clinically and experimentally, and perhaps Woolley's findings foreshadow such a development.

Another type of antithyroid effect is suggested by the findings of Pal

and Bose (1943). They found that the thyroid hyperplasia of rats fed on a poor diet was prevented by feeding *L*-tyrosine, suggesting that thyroid hormone synthesis was interfered with through a lack of tyrosine as building material. Other workers on phenylalanine- and tyrosine-deficient diets have not commented on the condition of the thyroid gland.

### *Summary*

In addition to various cellular and metabolic processes which it shares with other tissues, the thyroid gland has the unique capacity of accumulating iodide and using it to form thyroid hormone. All of these activities seem to be accelerated by thyrotropin from the pituitary. The administration of iodine, under certain experimental conditions, appears to depress the thyroid gland and to decrease the rate of formation of thyroid hormone. This peculiar effect is similar to that seen clinically when iodine is used in hyperthyroidism, and it remains wholly unexplained. When thyroid hormone is given, the thyroid gland is also depressed, but perhaps this is entirely a normal regulatory phenomenon mediated by the hypophysis.

Iodine metabolism within the thyroid gland, leading to the formation of thyroid hormone, can be divided into three major steps: (1) the concentration of iodide ion within the thyroid; (2) the oxidative conversion of iodide into an organic form, presumably diiodotyrosyl radicals of a protein; and (3) the oxidative coupling of pairs of diiodotyrosyl radicals to form thyroxyl groups.

The iodide-concentrating mechanism permits the thyroid gland to store iodide ion at a concentration several hundred times that of the circulating blood. Thiocyanate inhibits this. In the presence of thiocyanate, the gland is obliged to synthesize thyroid hormone from the iodide which passively diffuses into it from the blood. When the blood iodide is very low, because of deficient ingestion of iodine, hormone synthesis is deficient and hypothyroidism and goiter ensue.

The second major step in hormone synthesis, the oxidation of iodide, seems to be the site of action of antithyroid compounds of the thiocarbonamid and aminobenzene types. Presumably, some enzyme system is responsible for the oxidation of iodide. Of those proposed, one with the properties of a peroxidase seems the most likely. Compounds of the thio-sulfur type may inhibit this enzyme directly, compete for it by serving as a substrate, compete for the available hydrogen peroxide, or reduce the iodine as fast as it is formed. More must be known about how iodide is oxidized before the mechanism of inhibition can be more precisely defined.

The coupling of diiodotyrosyl groups to form thyroxyl radicals also requires an oxidation, but just how this is brought about is unknown. Thus far, no specific inhibitor for this conversion has been discovered.

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### *Discussion of the Paper*

DR. C. G. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*):

In connection with Dr. Astwood's reference to our early work, I would like to report that, while added dietary iodide inhibits by 50 per cent or more the thyroid enlargement produced by thiouracil on a normal diet, it does not do so with sulfanilamide, sulfadiazine, or sulfaguanidine. The response to PABA, on the other hand, is completely prevented by iodide or nearly so. In this respect, it resembles not the sulfanilamides but the thioureas. However, one can hardly assume, in the face of such different active groupings, that thiouracil and PABA inhibit the formation of thyroxin in the same way. In view of these results, it appears probable that we have at least three types of compounds, thioureas, sulfanilamides, and PABA, each of which blocks the formation of diiodotyrosine or thyroxin in a different way. This gives us three handles to grasp, three tools to employ, in attacking the series of chemical reactions involved in the synthesis of the hormone and its precursor in the body.

Second, it is of interest that, with thiouracil, one can produce almost any morphological response desired by manipulation of the levels of *iodide and the drug* in the diet. Thus, the size of the goiter can be reduced without changing the hyperplasia, or the hyperplasia may be suppressed without reducing the size of the goiter. (*Endocrinology*. XV: 137. 1944.)

Finally, I would like to make a plea for the use, wherever possible, of diets of known and preferably not excessive iodine content in all work on the thyroid, thyroxin, or TSH. This would greatly aid in advancing the field and permit more reliable comparison of results from different laboratories. A simple, purified diet of sugar, casein, yeast, fat, and salts could be used at least as a check on a few animals in any experiment.

DR. ISIDOR GREENWALD (*College of Medicine, New York University, New York, N. Y.*):

I wish to discuss two subjects mentioned by Dr. Astwood in his general summary. In the first place, no one has yet prevented the goitro-

genic action of a diet of low iodine content by adding such small amounts of iodine as are found in ordinary mixed diets. As Remington and Remington<sup>1</sup> wrote: "The technic (of Levine, Remington, and Kolnitz) must now be considered as of a curative rather than a preventive nature."

This statement applies equally well to all other attempts to counteract the goitrogenic effect of a particular diet. In every case, the amount of iodine required to produce thyroids of the small size found in the control animals was greater than that in the control diets, and the thyroids of the experimental animals contained more iodine than did those of the controls.

I also wonder just what we are to call a "normal" thyroid. Is a human thyroid weighing 95 grams "normal," as Oswald believed? Is a rat thyroid that shows almost complete absence of iodine and colloid "normal"? Yet animals with such thyroids could "reach maturity and produce normal numbers of living young."<sup>2</sup>

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# STUDIES ON THE METABOLISM OF THYROXINE IN THE BODY\*

By C. P. LEBLOND

*Department of Anatomy, McGill University, Montreal, Canada*

**I**N the past, the only available methods for following the fate of both thyroid preparations and crystalline thyroxine in the body were chemical determinations or biological assay of blood and tissues. While the biological activity of tissues after thyroid administration may give a rough estimate of the amount of thyroxine present, the chemical determinations were usually iodine titrations and thus estimated not only thyroxine, but other iodized compounds (iodide, diiodotyrosine). Furthermore, for the detection of thyroxine in tissues by either biological or chemical methods, a fairly large concentration of the material is required. In order to achieve this result, the dose of either thyroid extract or thyroxine had to be so large that it probably behaved in a manner quite different from the small amounts present under physiological conditions.

Today, the availability of radioactive iodine makes possible the study not only of large doses of thyroxine, but also of minute or physiological doses such as are handled normally in the body. In the study reported here, carried out in collaboration with J. Gross, both large and physiological doses of radiothyroxine were used. The large doses were prepared by iodination of diiodothyronine with radio-iodine; the physiological, by butyl alcohol extraction of the thyroxine fractions from the thyroids of rats previously treated with radio-iodine.

The preparation of large doses of radiothyroxine was first carried out by Horeau and Süe in 1945 according to a micro-technique adapted from the classical method of Harington and Barger for the synthesis of thyroxine. The technique, which consists in having free iodine react with diiodothyronine in alkaline solution, readily yields several milligrams of radioactive thyroxine which may be intravenously injected into rats in the form of the sodium salt.

The physiological doses were prepared as follows. Rats were treated with highly active radio-iodine (0.1 to 1 microgram of iodine containing 30 to 50 microcuries of radio-iodine). Twenty-four hours later, the thyroids of the animals were removed and dissolved in 2N sodium hydroxide. After measurement of their activity on the Geiger counter, the solutions were neutralized and extracted with *n*-butyl alcohol according to Blau's method, based on Leland and Foster's finding that thyroxine is preferentially taken up by butyl alcohol. Two further washings of the butyl extract with sodium hydroxide yielded a solution containing the radioactive thyroxine fraction. After evaporation of the butyl alcohol, the

\* This work was carried out with the help of a grant from the National Research Council of Canada.

thyroxine fraction was dissolved and injected into normal and thyroidectomized rats by the intravenous route. Since the amount of thyroxine injected was extracted from the thyroids of two 100-gm. rats, it may be assumed that less than 5 micrograms of thyroxine were administered (Taurog and Chaikoff, 1946). Since, furthermore, it is known that 5 micrograms of thyroxine are used up daily by the 100-gm. rat (Dempsey and Astwood, 1943), the dose administered was considered as a physiological one.

One of the most striking features of the action of thyroxine is the ability of this substance to produce a prolonged action in the body. A single injection of a large dose of thyroxine may maintain a higher than normal basal metabolic rate for several weeks. It was, therefore, expected that this substance would be detected in the body over a long period of time. However, the classical results obtained with large doses of thyroid extract or thyroxine showed that these substances disappear from the blood quite rapidly after their administration through various routes, as demonstrated in the case of thyroid extract by Abelin and Scheinfinkel (1925), Veil and Sturm (1925), Zawadowsky and Asimoff (1927), and in the case of thyroxine by Asimoff and Estrin (1931), Müller and Fellenberg (1932). Recently, this was further confirmed by Gaebler and Strohmaier (1942), who gave 10 mg. of thyroxine intravenously to dogs. Three minutes after injection, iodine titrations showed that only half of the dose was still present in the blood. Twenty-four hours later, over 90 per cent of it had disappeared from the blood. In this laboratory, even more rapid disappearance was observed with radiothyroxine, since, after an intravenous injection of about 2 mg., only 1.5 per cent of the injected amount, on the average, was found to remain in the blood (the blood volume being taken as 4.3 cc. per 100 gm.). Boe and Elmer found, with relatively smaller doses, such as 2 milligrams of thyroxine in man, that the organic blood iodine level returned to normal within 2 hours after injection.

Even with our minute physiological dose of several micrograms of radiothyroxine injected into the rat, the whole plasma contained only about 2 per cent of the administered dose 2 hours after injection.

It is interesting to note that, while thyroxine vanished from the blood rapidly in experiments carried out *in vivo*, this substance retained all activity when added to blood and incubated *in vitro* for 48 hours (Müller and Fellenberg, 1932). It may, therefore, be concluded that the disappearance of thyroxine from the circulation is due not to a local destruction, but to a withdrawal of thyroxine from the blood, presumably under the action of some other organ.

It was recognized early that the *liver* fixes large amounts of iodine after injection of thyroid extract. Furthermore, perfusion of the liver with a fluid containing thyroid extract resulted in a marked destruction of the thyroglobulin in this extract with formation of iodide (Blum and Grützner, 1920). Later, it was more accurately proven that, with large

doses of thyroxine, the liver takes up thyroxine from the blood and releases it into the bile (Kendall, 1919; Abelin and Scheinfinkel, 1925; Zawadowsky and Asimoff, 1927; Krayner, 1928; Asimoff *et al.*, 1931).

When large doses of radiothyroxine were administered (TABLE 1), the

TABLE 1  
DISTRIBUTION OF LARGE DOSES OF RADIOTHYROXINE IN THE RAT  
(Percentage of the injected dose, taken by the entire organ or tissue)

	2 Hours	24 Hours
<i>Plasma</i>		
(Total estimated volume)	1.51	0.16
<i>Internal organs</i>		
Liver	14.00	8.10
Lung	1.45	0.34
Pancreas	0.25	0.38
<i>Gastrointestinal tract</i>		
Stomach wall	0.72	0.11
Stomach contents	2.85	0.15
Duodenal wall	0.44	0.40
Duodenal contents	1.13	<0.01
Jejuno-ileal wall	7.94	0.47
Jejuno-ileal contents	30.10	0.18
Colon-caecum wall	1.00	0.14
Colon-caecum contents	4.20	0.61
Faeces	0.13	68.50
<i>Urinary tract</i>		
Kidney	1.35	0.29
Urine	3.40	19.70
<i>Skin</i>	3.70	1.35
<i>Muscle</i>	10.80	1.23
<i>Thyroid</i>	0.10	0.19

liver fixed within 2 hours as much as 10 to 40 per cent of the injected dose, with an average of nearly 20 per cent. Separation of the thyroxine fraction in this organ showed that most of the radioactivity present in the liver was still in the form of thyroxine, the balance being assumed to be iodide originating from the broken-down thyroxine. Similarly, Elmer and his collaborators carried out the chemical fractionation of the bile after thyroxine injection and observed the presence of a large amount of this substance as well as an approximately equal amount of iodide. This author concluded that half the thyroxine was broken down to iodide while passing through the hepatic cells on its way to the bile.

Only 2 animals have so far been treated with physiological doses. The amount of thyroxine taken up by the liver was considerably reduced, being on the average 4 per cent of the injected dose (TABLE 2). In this connection, it is interesting to note that Kellaway *et al.* (1945) found that physiological doses of thyroxine have the same effect in normal and hepatectomized rats, while doses 3 times as large were more active in hepatectomized than in normal animals. This observation suggested that the liver plays a greater role in the detoxification of larger doses of thyroxine than of the normally circulating physiological doses.

TABLE 2

DISTRIBUTION OF A PHYSIOLOGICAL DOSE OF RADIOTHYROXINE IN THE RAT  
(Percentage of the injected dose, two hours after injection)

<i>Plasma</i>	1.9
<i>Internal organs</i>	
Liver	4.4
Lung	0.6
Kidney	1.2
Pancreas	0.2
<i>Gastrointestinal tract secretions</i>	
Stomach content	8.5
Duodenal content	0.3
Jejuno-ileal content	4.0
Colon content	1.9
<i>Endocrine glands</i>	
Thyroid	0.5
Hypophysis	tr.
Adrenals	tr.
<i>Muscles</i>	7.1
Skin	5.1

In the *endocrine glands*, it has not been possible with physiological doses of radiothyroxine to confirm in the rat the claims of a specific fixation of thyroxine in the hypophysis as found in other species by Sturm and Schneeberg (1933) and by Joliot *et al.* (1944). In the thyroid gland, there is little radioactivity soon after administration of radiothyroxine, a fact which shows that this hormone does not normally enter the gland.

Krayer (1928) and Müller, and Fellenberg (1942) described large amounts of thyroxine in the skin. In this laboratory, it was found that a fair amount of radiothyroxine was present in the whole skin of the animal (TABLES 1 and 2), but the concentration was rather low.

A study of the excretion organs showed that the gastrointestinal tract had a much more important role than the kidney. The kidney, after the administration of thyroxine or thyroid extract, contained an appreciable amount of the active substance (Kendall, 1913; Asimoff and Estrin, 1931). However, the iodine in the *kidney and urine* was found to be in the form of iodide (Veil and Sturm, 1925; Zawadowsky and Asimoff, 1927), suggesting that thyroxine itself was hardly eliminated in the urine at all. It will be seen, in TABLE 1, that, 24 hours after administration, the excretion in the urine makes up close to a third of the injected dose.

Important connections were found between the thyroid hormone and the *gastrointestinal system*. This observation was rather surprising owing to the scarcity of references on the subject in the literature. It was found that, with large doses of radiothyroxine, a high proportion of the injected dose was present in all the sections of the gastrointestinal tract (TABLES 1 and 2). However, partition studies have shown that the radioactivity in the stomach contents are to a large extent in the non-thyroxine fraction, while the radioactivity was predominantly in the thyroxine fraction in the small and large intestine. Finally, following the ligation

of the bile duct, pylorus, duodeno-jejunal and ileo-coecal junction, much greater amounts of radioactivity were present in the gastric and duodenal contents than in the contents of the jejuno-ileum or large intestine. It was concluded that a marked excretion of thyroxine as such takes place in the duodenum, while the radioactivity found in the stomach testifies to the excretion of breakdown products of thyroxine (iodide?).

Since, on the other hand, it is known that thyroxine deposited in a ligated intestinal loop is rapidly absorbed (Schittenhelm and Eisler, 1932), it appears likely that a large proportion of the thyroxine excreted into the duodenum is resorbed in the lower portions of the intestine. However, 24 hours after the injection of a large dose of thyroxine, a high proportion of it was found in the feces, namely, over two-thirds of the injected dose. The feces are, therefore, the main channel of excretion of thyroxine.

### *Summary*

Thyroxine is swiftly withdrawn from the blood. This substance is distributed in the body, as indicated in TABLE 1. The liver and especially the gastrointestinal tract play an important role in thyroxine metabolism.

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*Discussion of the Paper*

DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

An item of great interest in Dr. Leblond's studies is the fact that sizable quantities of extraneous radioactive thyroxine reach the tissues as thyroxine. We may assume, from this, that the same is true for endogenous thyroxine released into the circulation from the native hormonal complex of the thyroid. At the same time, the quick discharge of radioactive thyroxine into the liver and gastrointestinal tract, after the administration of large doses, is also of interest. One wonders what the limiting factor is in the various tissues for holding adequate and safe amounts of thyroxine. In other words, how great is the "thyroxine hunger" of tissues?

Is it known whether radioactive iodine, when administered as such, has the same or a different tissue distribution coefficient as radioactive thyroxine? And does one know anything of a possible correlation between the "normal" (the average, as recorded) iodine level in various tissues and their retention of administered iodine or thyroxine? It is known that tissues contain iodine, even when the supply of  $I_2$  to the thyroid is inadequate.

DR. ALBERT J. RITZMANN (*Brooklyn, N. Y.*):

Dr. Leblond's method of using tagged thyroxine demonstrates the dispersion of the hormone systemically with findings of comparatively large amounts in some organs such as the muscle, liver, skin, heart, and gastrointestinal tract, as compared to relatively small amounts in the central nervous system and other tissues. These findings can be carried over clinically. In patients with marked hyperthyroidism, in diffuse toxic goiter, the abnormal or exaggerated findings of excess skin perspiration, irritable heart, diarrhea, altered liver function and aimless, exaggerated muscle movements link up with those organs or tissue systems in which the thyroxine content is high. Interpreted in another way, Dr. Leblond's findings show the association of high thyroxine concentration in tissues or organs where functional need and cell metabolism are greatest.

# THE FORMATION OF THYROXINE IN IODINATED PROTEINS

By E. P. REINEKE

*Department of Physiology and Pharmacology, Michigan State College,  
East Lansing, Michigan*

THE iodination of proteins has been the subject of investigations covering a period of half a century. In many respects, progress in this field has gone hand in hand with the development of knowledge concerning the natural secretion of the thyroid hormone. Immediately following the discovery of iodine in organic combination in the thyroid by Baumann in 1895,<sup>1</sup> investigations were conducted to determine whether a thyroidal substance could be formed by the simple iodination of proteins. This early work has recently been reviewed in detail by Salter<sup>2</sup> and Reineke.<sup>3</sup> Although the early attempts were not successful in the formation of an active product, considerable information was gained on the types of combination of iodine with proteins, and the methods by which the combination could be effected. Of the various iodination methods employed, the most noteworthy is that of Blum and Vaubel,<sup>4</sup> who buffered their protein solutions with sodium bicarbonate in order to neutralize the hydriodic acid that forms as a side product from the substitution of iodine. With proper control of conditions, this method can now be used for the formation of iodinated proteins possessing marked thyroidal activity.

In the light of present knowledge, it is difficult to evaluate early claims of thyroidal activity in iodinated proteins, since the biological assay methods employed were not well standardized, nor were the conditions employed in forming these substances fully controlled. Blum<sup>5</sup> reported that his iodinated albumin produced curative effects in myxedema, but this claim was later withdrawn.<sup>6</sup> Following the discovery that thyroid substance will accelerate the metamorphosis of frog tadpoles,<sup>7, 8</sup> it was reported that comparable effects were produced with iodinated proteins.<sup>9-11</sup> Alkaline hydrolysis was reported to destroy the activity of such preparations.<sup>12</sup> Since we now know that thyroxine is relatively resistant to destruction during alkaline hydrolysis, it appears unlikely that it had actually been formed in these preparations. In fact, the effects observed were considered at the time as merely indicating a special reaction of such preparations on tadpoles, and not a true thyroidal effect.

*Thyroidal Activity of Iodinated Proteins and Their Hydrolysates.* With the isolation of thyroxine by Kendall<sup>13</sup> and its synthesis by Harington and Barger,<sup>14</sup> it appeared extremely unlikely that a compound of this nature could be formed simply by the iodination of proteins. Conse-

quently, it is not surprising that subsequent results suggesting such a possibility were viewed with some skepticism.

However, Brandt, Mattis, and Nolte<sup>15</sup> reported that an acid-insoluble precipitate obtained from an iodinated protein after hydrolysis with barium hydroxide exerted a thyroid-like action on frog tadpoles. Likewise, Abelin and co-workers presented a series of reports<sup>16-21</sup> in which they gave convincing evidence that the acid-insoluble concentrates obtained from iodinated proteins after hydrolysis with alkali produced many effects that were qualitatively indistinguishable from those elicited by thyroxine.

The isolation from iodinated proteins of thyroxine in crystalline form was finally reported by Ludwig and von Mutzenbecher<sup>22</sup> and confirmed by Harington and Pitt Rivers.<sup>23</sup> The importance of conducting the iodination process under exact, but rather empirically selected conditions was emphasized. Little attention was given, however, to the possible activity of iodinated proteins prior to hydrolysis or the influence of varying the reaction conditions on the activity of the resulting product. Although it had been reported<sup>16, 17</sup> that iodinated proteins produced thyroidal effects only after hydrolysis, the reports of Kaer,<sup>24</sup> Lerman and Salter,<sup>25</sup> Harington and Pitt Rivers,<sup>23</sup> and Reineke and Turner<sup>26</sup> indicated that some whole iodinated proteins produce significant thyroidal effects. Consequently, our attention was turned to the possibility of increasing the activity of iodinated proteins by suitable control of the reaction conditions.

*Factors Affecting the Formation of Active Substance.* In the subsequent investigations, the procedure was adopted of varying single factors in the iodination and incubation processes while maintaining other conditions constant in so far as possible. Until the applicability of chemical methods for the determination of thyroxine in such preparations had been established<sup>27</sup> biological assay methods were employed to determine their thyroidal potency.

In making the preparations, 20 gm. of casein was placed in 700 ml. of distilled water containing sodium bicarbonate, and dissolved by stirring. The solutions were then placed in a constant-temperature water bath, and finely powdered iodine was added slowly over a period of 3 to 4 hours with vigorous stirring. The mixture was then incubated for a period of 18 to 20 hours at constant temperature, the stirring being continued throughout the process. The solutions were finally dialyzed and the iodinated protein was recovered by isoelectric precipitation.

When the sodium bicarbonate added in the above procedure was varied over a broad range in succeeding preparations,<sup>28</sup> good potency was observed as long as the bicarbonate used was sufficient to maintain a pH of 7.0 or above. Even though normal amounts of iodine were combined at lower pH values, there was a pronounced decline in thyroidal activity.



By a similar procedure, it was found that the amount of iodine added has a controlling influence on the amount of thyroidal substance formed. The potency of the iodinated protein increased progressively with increasing iodine input until 4.5 to 5.0 atoms of iodine had been added per mole of tyrosine in the protein.<sup>29, 30</sup> Iodination beyond this point resulted in pronounced decreases of activity. In the medium employed, only one-half of the reacting iodine is substituted on the tyrosine radical, the remainder being used to form hydriodic acid. Consequently, the optimal iodine input under these conditions would be slightly in excess of the amount required to substitute two atoms per mole of tyrosine in the protein. The excess would be available for oxidation in the coupling of two molecules of diiodotyrosine to form thyroxine. With a greater excess of bicarbonate, more iodine was required to reach the point of maximum potency.<sup>31</sup>

Proteins iodinated in a more alkaline ammoniacal medium by Muus *et al.*<sup>31</sup> did not reach their peak activity until considerably more iodine had been combined, and failed to show a decline in potency with excessive iodination. In the opinion of the author, this difference in results can be explained by the fact that the reactivity of diiodotyrosine as well as the oxidative action of iodine declines with increasing alkalinity of the medium.<sup>32</sup>

In all the earlier studies, the iodination and incubation procedures were conducted at physiological temperature on the assumption that this temperature would be optimal for thyroxine formation. Further investigation revealed that quite the reverse was true. When the temperature was increased to 60° to 70°C. during either the iodination or incubation steps and maintained at the elevated level for 18 to 20 hours,<sup>28</sup> a pronounced rise in the thyroidal potency of the resulting product occurred. At temperatures in excess of 90°C., little active substance was formed.

Two additional factors were found<sup>30</sup> to influence significantly the formation of active iodinated proteins, namely, the amount of stirring or aeration and the inclusion of any one of a series of manganese compounds as a catalyst (TABLE I). With other conditions held constant, there is a considerable increase in the apparent thyroxine content when the amount of agitation is increased sufficiently to whip air into the solutions. Still another increase in potency occurs if the incubation is conducted in the presence of a manganese compound. Under the conditions employed, manganese tetroxide ( $Mn_3O_4$ ) and the oxides obtained by the reduction of potassium permanganate with glucose exerted the greatest effect.

The combined influence of several interacting factors on thyroxine formation is shown in FIGURE 1. Manganese tetroxide appears to be effective over a considerable range of iodine concentrations. Further, the amount of iodine added remains a critical factor in the presence of manganese.

TABLE I

EFFECT OF INCUBATION TEMPERATURE, MANGANESE COMPOUNDS, AND AMOUNT OF AGITATION ON FORMATION OF THYROXINE IN IODINATED PROTEIN  
(From *J. Biol. Chem.* 161: 613. 1945.)

<i>Catalyst</i>	<i>Stirring r.p.m.</i>	<i>Thyroxine content per cent</i>	<i>Average per cent</i>
Series I. Skim milk proteins iodinated and incubated at 37°			
None	Very gentle	0.33	
None	Very gentle	0.26	
None	Very gentle	0.27	0.29
Series II. Casein iodinated at 38–40°, incubated at 70°			
None	300	1.67	
None	600	1.73	
None	600	1.80	
None	600	1.75	
None	600	1.84	1.76
Mn <sub>2</sub> O <sub>4</sub>	300	1.94	
Mn <sub>2</sub> O <sub>4</sub>	300	1.99	1.96
Mn <sub>2</sub> O <sub>4</sub>	600	2.72	
Mn <sub>2</sub> O <sub>4</sub>	600	2.93	
Mn <sub>2</sub> O <sub>4</sub>	600	3.03	
Mn <sub>2</sub> O <sub>4</sub>	600	2.78	
Mn <sub>2</sub> O <sub>4</sub>	600	2.80	
Mn <sub>2</sub> O <sub>4</sub>	600	3.04	2.88
Oxides from reduction of KMnO <sub>4</sub>	600	2.97	
	600	2.96	
	600	2.60	2.84
MnO <sub>2</sub>	600	2.16	
MnO <sub>2</sub>	600	2.19	2.17
Mn <sub>2</sub> O <sub>3</sub>	600	2.26	
Mn <sub>2</sub> O <sub>3</sub>	600	2.33	2.30
MnSO <sub>4</sub>	600	2.00	
MnSO <sub>4</sub>	600	2.13	2.07

Although it is possible to demonstrate some thyroidal activity in iodinated proteins prepared under a variety of conditions, all of the factors discussed appear to be critical and must be maintained at the optimum in order to obtain preparations of high potency.

Further information on the control of thyroxine formation is provided by results obtained in the direct synthesis of thyroxine from diiodotyrosine. It was first reported by von Mutzenbecher<sup>33</sup> that, when diiodotyrosine is incubated in mildly alkaline solution at 37°C. for a period of two weeks, crystalline thyroxine equivalent to about 0.1 per cent of the diiodotyrosine used initially is formed. This was fully confirmed by others<sup>34–36</sup> using an identical procedure. Harington<sup>37</sup> stated that, when diiodotyrosine was oxidized with hydrogen peroxide on the steam bath, the solution meanwhile being shaken constantly with *n*-butanol to extract the thyroxine as it was formed, a greatly improved yield was obtained.

When diiodotyrosine was dissolved in N/10 sodium hydroxide and

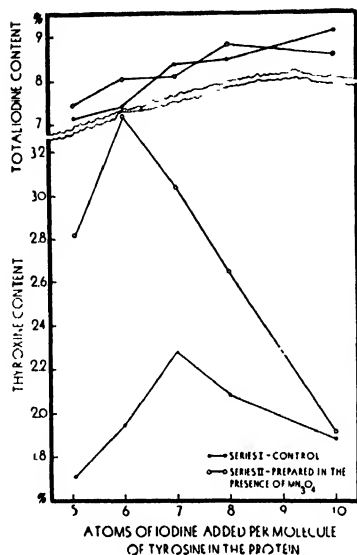


FIGURE 1. The effect of progressive iodination in the presence of excess bicarbonate and manganese oxide on the thyroxine content of iodinated casein. (From *J. Biol. Chem.* 161: 613. 1945.)

incubated for 18 to 20 hours with vigorous stirring or aeration,<sup>32</sup> the results were very similar to those obtained with iodinated casein (FIGURE 2). Quite appreciable yields of thyroxine were obtained under optimum conditions, amounting to 0.85 per cent of the diiodotyrosine incubated. If allowance was made for the unaltered diiodotyrosine that could be recovered, the net yield amounted to 2.8 per cent. The incubation temperature is highly critical, with an optimum at about 60°C. Thyroxine

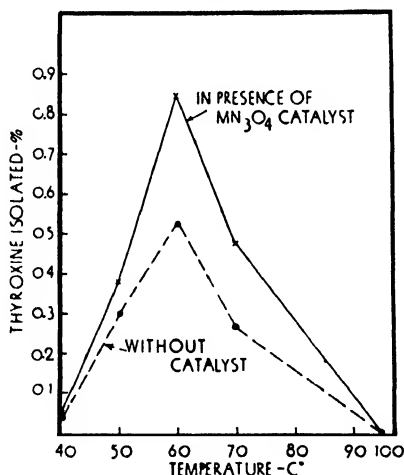


FIGURE 2. Gross yield of thyroxine isolated after incubation of diiodotyrosine at various temperatures. (From *J. Biol. Chem.* 162: 369. 1946.)

formation was increased somewhat in the presence of manganese tetroxide at all points in the effective temperature range. In addition, it was found that thyroxine formation was negligible unless the solutions were either stirred or aerated directly, and also that manganese tetroxide catalyzed the reaction only when air was introduced. It appears probable, therefore, that the effect of manganese is due to its acceleration of an atmospheric oxidation taking place at some point in the chemical system involved. Further investigation will be required to determine the site of action.

*Nature of the Active Substance in Iodinated Proteins.* From the fact that thyroxine can be isolated readily from iodinated proteins prepared under suitably controlled conditions, there can be no doubt that their thyroidal activity is due at least in part to their thyroxine content. Ludwig and von Mutzenbecher,<sup>22</sup> as well as Harington and Pitt Rivers,<sup>23</sup> reported that subsequent to hydrolysis of iodinated proteins with barium hydroxide approximately 0.1 per cent of crystalline thyroxine was recovered.

By the use of highly active iodinated casein and a similar method of hydrolysis and isolation, Reineke and Turner<sup>38</sup> obtained a yield of 0.424 per cent of crystalline *DL*-thyroxine. In a more recent attempt by the author (unpublished), 0.5 per cent of thyroxine was isolated. The identity of the compound is fully established by its characteristic crystalline structure (FIGURE 3), by the fact that it shows an ultraviolet absorption curve identical with that of synthetic thyroxine (FIGURE 4), by its iodine content of 64 to 65 per cent, and finally by its high metabolic potency when administered to test animals.

The maximum yield isolated actually represents about 5 times the thyroxine content of USP thyroid. However, it is only about 1/6 of the amount of thyroxine that is apparently present, as judged from the results of chemical and biological assays. The discrepancy can be accounted for, in part, in the sizable losses of thyroxine involved in its isolation and purification. In addition, there are apparently variable losses during the hydrolysis in strong boiling barium hydroxide solution that is required to liberate the thyroxine. There is also the possibility that a part of the activity is due to the presence of an as yet unidentified thyroxine-like compound.

As would be expected from its method of formation, thyroxine exists in iodinated proteins in the natural *L*-form. In the usual alkaline hydrolysis, racemization occurs, so that a *DL*-mixture is obtained. When active iodinated protein was hydrolyzed in a mixture of sulfuric acid and *n*-butanol, racemization was avoided and pure levorotatory thyroxine was isolated quite readily.<sup>39</sup>

The relative potency of *L*-thyroxine compared with that of the racemic mixture that is more easily available for use as a standard, is of considerable importance in evaluating the results of biological assays. Biological

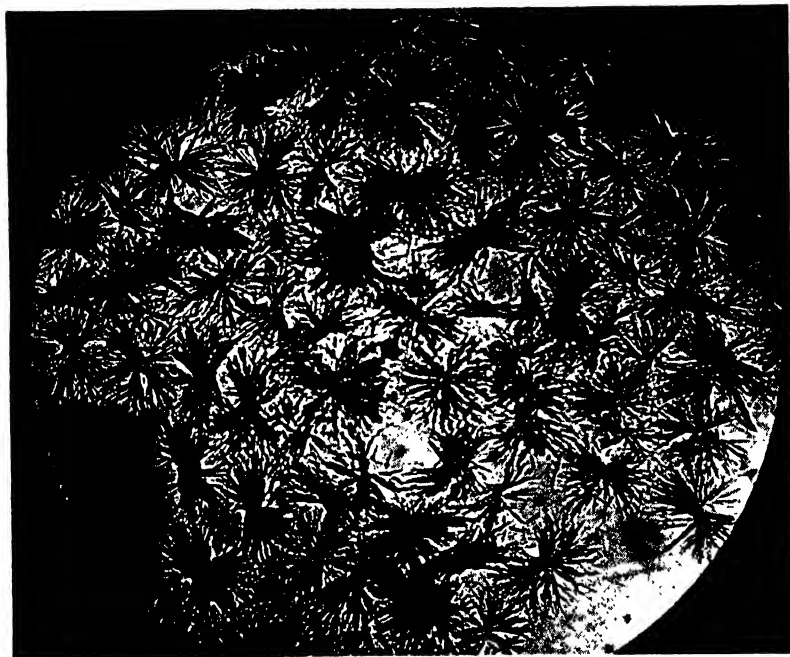


FIGURE 3. Spectrographic absorption curves of synthetic thyroxine and thyroxine isolated from a barium hydroxide hydrolysate of iodinated casein. (From J. Biol. Chem. 149: 555. 1943.)

assays of the compounds separated by Harington from a racemic mixture indicated<sup>40-42</sup> that *d*-thyroxine has 1/3 or more of the activity of the *L*-form. However, it was pointed out in the original report on the resolution of these compounds<sup>43</sup> that the separation of isomers was probably not complete. The higher specific rotation of *L*-thyroxine tested more recently<sup>39,44</sup> indicates better optical purity. Thus, it seems that the activity attributed to *d* thyroxine could be accounted for by contamination with *L*-isomer. It should be noted, however, that Deanesly and Parkes<sup>45</sup> tested on *Xenopus* tadpoles a specimen of synthetic *L*-thyroxine that showed a high specific rotation, and failed to find it more active than the *dl*-mixture.

The chart shown in FIGURE 5 is typical of results we obtained<sup>46</sup> when the potency of *L*-thyroxine isolated from iodinated casein was compared with that of a *dl*-mixture. In this instance, the ability of the thyroxine to prevent the increase in the thyroid weight of thiouracil-treated chicks was used as the measure of response. When the data are plotted so that the *dl*-thyroxine dosage scale is twice that used for *L*-thyroxine, the response curves are identical, demonstrating that the latter preparation has twice the activity of the former. Similar results were obtained in tests on thiouracil-treated rats, by the metabolic stimulation of guinea pigs, and by the metamorphosis-stimulating effect in *Rana pipiens* tad-

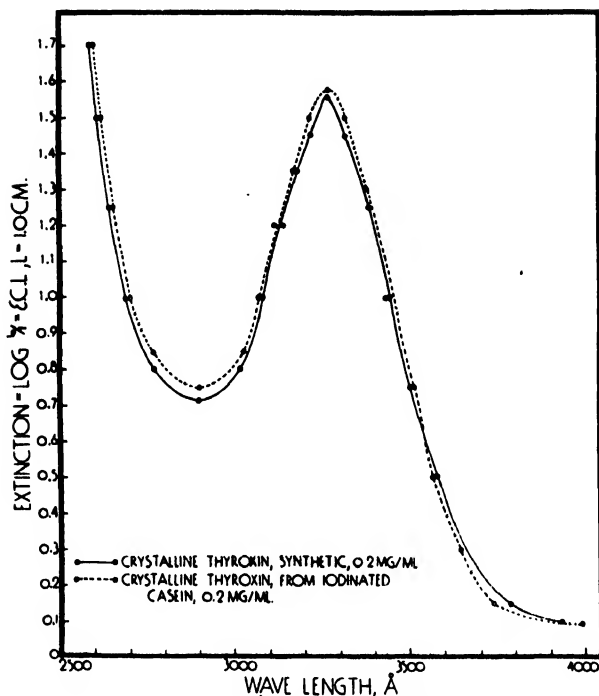


FIGURE 4. Crystalline *dl*-thyroxine isolated from thyroactive iodinated casein subsequent to hydrolysis with barium hydroxide ( $\times 400$ ).

poles. Thus, it was concluded, in agreement with Foster *et al.*,<sup>44</sup> that all of the activity of *dl*-thyroxine can be accounted for by its *l*-component. In attempting to determine the thyroxine content of iodinated proteins by biological assays, comparisons have either been made with an *l*-thyroxine standard, or, where a *dl*-standard was used, the apparent thyroxine content of the preparation under test has been divided by two to convert the value to the *l*-thyroxine basis. The biological assay of thyroidal preparations is further complicated by differences in the absorption of different substances, particularly when administered orally. Quite surprisingly, it was found<sup>28</sup> that, when dissolved in mildly alkaline solution, iodinated proteins are highly effective when given by injection. Therefore, in attempting to estimate the active substance actually present in the iodinated protein, it was injected and the response compared directly with that obtained with injected thyroxine.

The specificity of chemical methods for the determination of thyroxine, when applied to iodinated proteins, must be well established before the results obtained can be interpreted properly. Measurement of the acid-insoluble iodine of iodinated protein hydrolysates provides only a rough index of potency because considerable amounts of non-thyroxine iodine are included in this fraction.<sup>45</sup> Preliminary results with Blau's

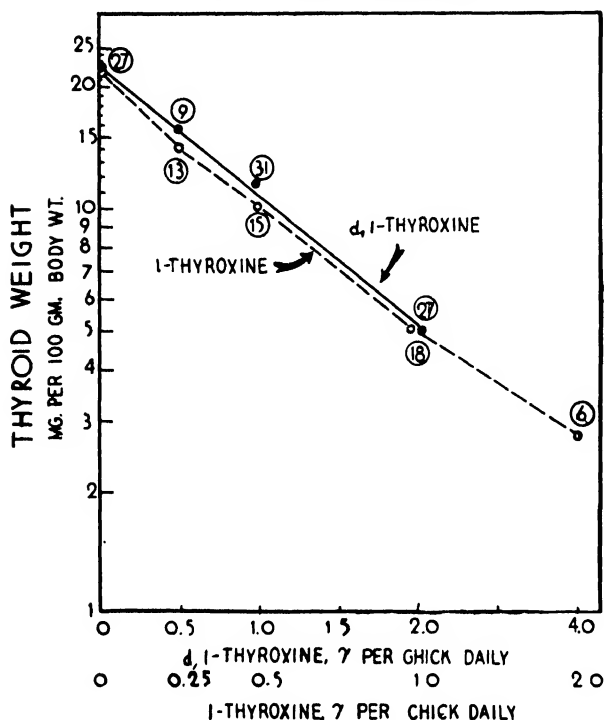


FIGURE 5. The relative potency of *l*- and *dl*-thyroxine in reducing the thyroid weight of thiouracil-treated male chicks. The encircled numerals indicate the number of animals per dosage group. (From *Endocrinology* 36: 200, 1945.)

*n*-butanol extraction procedure for the determination of thyroxine in thyroid substance failed to give good agreement with biological assays when applied to iodinated proteins. When a more vigorous hydrolysis was used, however, excellent agreement was found (TABLE 2) between the chemical thyroxine analysis and the bioassay value obtained by determining the metabolic response to intraperitoneally injected iodinated protein. The biological assay method yielded values that averaged about 8 per cent below the chemical method. This difference might be accounted for either by the inclusion of a small amount of non-thyroxine iodine in the chemical determination, or by a lower absorption, from the injection site, of the iodinated protein than of the thyroxine used as a standard. By suitable control of conditions, iodinated proteins that apparently contain about 3 per cent of thyroxine as determined by either method can now be prepared quite consistently. Biological assays made at various stages of the *n*-butanol extraction procedure indicated that all the active substance in the iodinated protein is recovered in the extraction process.

Even though close agreement is obtainable between the chemical and biological methods, the possibility of the occurrence in iodinated pro-

TABLE 2

DATA DEMONSTRATING THE CORRELATION BETWEEN THE CHEMICAL AND BIOLOGICAL  
ASSAY METHODS FOR THYROXINE(From *J. Biol. Chem.* **161**: 599, 1945.)

Preparation No.	Iodine added per mole tyrosine in protein, atoms	Iodinated protein injected, %100 g. body wt.	Increase in CO <sub>2</sub> output, per cent	Thyroxine found		Difference, per cent
				Bioassay,* per cent	Chemical analysis, per cent	
1		223	25.4	2.46	2.69	-8.6
2		138	24.7	3.80	3.91	-2.8
3		176	20.8	2.46	3.06	-19.6
4		300	27.6	2.01	2.06	-2.4
5		150	20.9	2.90	3.88	-25.3
6		145	25.1	3.71	3.73	-0.5
6		161	25.0	3.31	3.73	-11.3
6		161	25.1	3.34	3.73	-10.5
7	4.51	243	21.7	1.86	2.21	-15.8
8	5.01	198	23.4	2.50	2.71	7.7
9	5.51	201	26.8	2.90	2.69	+7.8
10	6.01	190	23.0	2.55	2.83	-9.9
11	6.51	175	23.1	2.78	3.09	-10.0
12	7.01	174	22.2	2.67	3.11	-14.1
13	8.01	191	26.4	2.98	2.83	+5.3
14	9.01	194	23.2	2.53	2.78	-9.0
15	10.01	209	25.8	2.66	2.58	+3.0
Weighted average				2.79	3.04	-8.1

\* Estimated from standard response curve for intraperitoneally injected *L*-thyroxine.

teins of an active compound other than thyroxine is not wholly excluded. Such a compound, if present, however, would need to have a thyroidal activity per unit of iodine that is very similar to that of thyroxine iodine. On the other hand, if all of the substance measured by these methods is actually thyroxine, it should be possible to isolate more than the 0.5 per cent yield thus far recovered subsequent to hydrolysis.

Thyroactive iodinated proteins are effective when given orally in all species in which they have been tested to date. The oral effectiveness is considerably less than by injection and will probably vary in different species. Reineke and Turner<sup>47</sup> reported that iodinated protein was only about 5 per cent as effective when given to sheep orally as by subcutaneous injection. Even thyroxine given in alkaline solution was only about 12 per cent as effective by oral as by parenteral administration in this species. Deanesly and Parkes<sup>48</sup> point out that iodinated proteins apparently are not utilized as effectively as thyroid substance when administered orally to cattle. Species with a simple digestive system appear to utilize the active substance in iodinated proteins far more effectively than do ruminant animals. However, exact comparisons of the relative oral potency of iodinated protein, thyroid substance, and thyroxine in other species, including man, have not been reported.

*Mechanism of Thyroxine Formation.* When publishing their classical experiments on the constitution and synthesis of thyroxine, Harington



and Barger<sup>14</sup> proposed the theory that thyroxine is synthesized *in vivo* by the iodination of tyrosine, followed by the oxidative coupling of two molecules of diiodotyrosine and the elimination of one side chain.

With the now well-established finding that thyroxine can be formed simply by the incubation of diiodotyrosine, or by the iodination of proteins under suitable conditions, it is proved beyond a doubt that this overall reaction will take place quite readily even without the intervention of a biological enzyme system.

However, there is little experimental evidence on the actual mechanism involved. By analogy with the findings of Pummerer *et al.*<sup>49</sup> on the oxidation of *p*-cresol, Johnson and Tewkesbury<sup>35</sup> proposed a series of reactions that would account for the oxidative conversion of thyroxine to diiodotyrosine (FIGURE 6). This would involve the oxidative coupling of

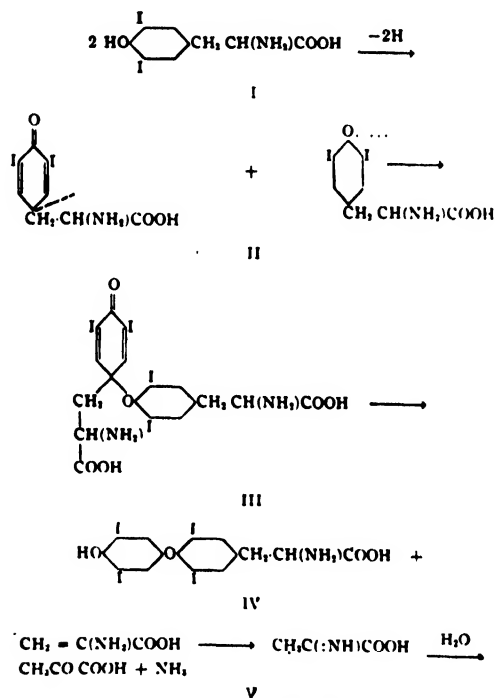


FIGURE 6. A mechanism for the conversion of diiodotyrosine to thyroxine. (From Proc. Nat. Acad. Sci. 28: 73. 1942.)

two molecules of diiodotyrosine (I and II) to form the intermediate compound III. Compound III could follow one of two pathways, namely, (a) molecular dissociation with loss of one alanine side chain and formation of thyroxine, IV, and aminopyruvic acid, or (b) hydrolysis, with production of serine. Qualitative tests indicated the presence of pyruvic acid and ammonia, but not serine, as secondary products in the reaction mixture. Harington<sup>28</sup> developed still further the theoretical background sup-

porting this theory. However, no actual evidence has been obtained on the intermediate compounds that would be necessary to fully confirm the proposed mechanism.

All the facts established thus far are consistent with the view that in the iodination of proteins, thyroxine is formed *within the protein molecule* by the oxidative coupling of two diiodotyrosine radicals, and the subsequent splitting-off of one side chain.

Although iodine can be substituted on the tyrosine combined in proteins under a variety of conditions, the secondary reactions involved in the formation of thyroxine are dependent upon the maintenance of mildly oxidative conditions in the reaction medium. It has been suggested by a number of workers that the coupling reaction involved is actually effected by the oxidative action of iodine. It is now established that thyroxine formation can be accelerated by incubation at 60° to 70°C. in the presence of vigorous stirring or aeration. The catalytic effect of manganese is apparent only in the presence of oxygen. It can easily be demonstrated that all of the iodine added combines either with the protein or as iodide during the iodination step. Consequently, no free iodine would be present for oxidative purposes during the incubation period when most of the thyroxine is formed. If we accept the idea that iodine is the effective agent in the conversion of diiodotyrosine to thyroxine, the influence of oxygen and manganese at high incubation temperatures might be explained by their oxidation of iodide to a more highly oxidized form such as hypoiodite. This compound would then be available for the oxidative coupling reaction. From the evidence now available, it seems clearly established that the thyroxine formed during iodination remains in firm combination in the protein molecule. Quite a vigorous hydrolysis with either alkali or acid is required to liberate the thyroxine so that it may be isolated in crystalline form. Furthermore, no loss of potency is observed after long-continued dialysis of the iodinated protein, indicating again that the thyroxine is combined in a large non-dialyzable molecule.

Our knowledge of protein structure is, of course, too meager to justify much speculation on the mechanism whereby two diiodotyrosine radicals, both of which are already combined in a protein molecule, could undergo the coupling reaction involved in the formation of thyroxine. It seems reasonable to believe, however, that only a certain proportion of the diiodotyrosyl radicals would be arranged spatially in such a position that they could undergo the reaction.

It can be calculated, for example, that in casein containing 5.65 per cent of tyrosine the theoretical thyroxine yield, if all of the tyrosine were iodinated and subsequently converted to thyroxine, would be about 10.6 per cent. Iodinated casein containing slightly more than 3 per cent of thyroxine-like substance, as determined by chemical analysis, can be prepared quite consistently, but no method of treatment has been found that will increase the conversion much beyond this point. The formation

of additional thyroxine may, then, be impossible because of spatial incompatibilities.

Many parallels could be drawn between the formation of thyroxine in iodinated proteins and in the thyroid gland. In both instances, it appears well established that the synthesis consists, first, of the substitution of iodine on the tyrosyl radicals of the protein, and, secondly, the oxidative coupling of two diiodotyrosyl radicals within the protein molecule to form a thyroxyl radical.

Both reactions will take place in proteins iodinated artificially, without the intervention of enzymes. In the process occurring *in vivo*, it would presumably be necessary to have an enzyme system capable of oxidizing iodide to iodine to permit its substitution on tyrosine. Whether the oxidative coupling reaction involved in the formation of thyroxine from diiodotyrosine is actually effected by hypiodite or a more highly oxidized form of iodine in either iodinated proteins or the thyroid, remains to be established by further investigation.

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### *Discussion of the Paper*

DR. SAMUEL DVOSKIN (*Columbia University, New York, N. Y.*):

The experiments of Dr. Reineke have clearly elucidated the optimal conditions under which thyroxine is formed *in vitro* by the iodination of proteins. It may be of interest to present the results of recent experiments with the rat and chick, in which elemental iodine was injected subcutaneously *in vivo*.

Injections of a solution containing elemental iodine served to reinitiate bone growth and increase body length in young thyroidectomized female rats. Equivalent dosage of iodide only exerted minimal effects. In normal young female rats, the elemental iodine injections caused a marked decrease in cell height of the thyroid epithelium and a decrease in the weight of the thyroid gland. Iodide injections, in equivalent dosage, failed to alter gland weight and only slightly lowered cell height. In thiouracil-fed chicks, or thiouracil- or sulfadiazine-fed young female rats, the subcutaneous injection of a solution containing elemental iodine caused a complete inhibition of the goitrogenic effects. Iodide injections, in equivalent dosages, failed to decrease the thyroid cell height, but partially reduced the thyroid gland weight.

Oral administration of solution containing elemental iodine was no more effective than oral administration of iodide solutions in influencing thyroid weight and structure in normal or thiouracil-fed rats.

From the evidence, it appears that the subcutaneous administration of elemental iodine to the rat and chick has an action similar to thyroxine. It is possible that the action of elemental iodine results from the formation of an iodoprotein, *in vivo*, having thyroxine-like action. However, no evidence is at hand at present to prove this hypothesis.\*

DR. VICTOR M. TRIKOJUS (*University of Melbourne, Melbourne, Australia*):

Since the lactic acid analogue of 3:5-diiodotyrosine has been shown by Foster and Gutman to be a metabolite of the amino acid, and Saul and Trikojus<sup>1</sup> have demonstrated its conversion by incubation to the corresponding analogue of thyroxine, it is pertinent to ask whether such a transformation would be possible in the body. Salter<sup>2</sup> has referred to the presence of a substance in hydrolysates of iodinated serum protein similar to thyroxine as regards its iodine content, but being nitrogen-free.

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DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

The new results which Dr. Reineke obtained in several species, amphibian, avian, and mammalian, when assaying his various iodinated proteins for thyroid-hormone activity, are very impressive. The formation in such proteins of the actual hormone, thyroxine, is now well understood. Very early, experimenters had realized the necessity of iodine for the physiological activity of the thyroid hormone, and especially the importance of the combination of iodine in organic form. Some investigators, however, maintained that the tadpole effect could be elicited by uncombined inorganic iodine. These views were found to be erroneous, for it was soon recognized that the animals treated with I<sub>2</sub> were maintained on protein foods, the latter being fed in association with iodine. Iodized peptides also elicited a semblance of the tadpole effect. Further, the possibility (almost certainty) that some inorganic iodine, after entering the tadpole, would become organically combined, could not be ruled out.

In 1916/17, we carried out numerous series of tadpole experiments, later interrupted by the War, with fractionated thyroid derivatives (nucleoproteins, globulins, coagulable proteins, etc.). These elicited the typical thyroid effect, albeit in varying degrees, nucleoproteins being the most potent ones. Unfortunately, we equalized the concentrations used according to the nitrogen, not the iodine, content of the fractions. However, the connection between iodine content and activity was readily seen. When graded according to their iodine content (mg./cc.), the fractions lined up exactly as when arranged according to hormonal potency. Yet, "it is not the iodine itself which provides this activity, but the

\* Dr. S. Barker has recently presented evidence that iodoproteins are formed at the injection site.

special coupling of iodine with some particular protein fraction, the most potent combinations being those present in the thyroid. Thyroxine contains only twice as much iodine per molecule as diiodotyrosine, yet it is not just twice but several hundred times as active as the latter. As an example, tadpoles will respond to a determinable minimum quantity of thyroxine, but when we treat them with twice the quantity of diiodotyrosine they will show no thyroid response whatsoever, though getting the same amount of iodine. We would have to apply a much higher (more than a thousand times higher) concentration of diiodotyrosine. Likewise, tetra-iodothyronine (thyroxine) with its four I atoms is far more than twice as potent as diiodothyronine with two I atoms, though otherwise the molecules are similar.”\*

The effectiveness of iodine when coupled with protein constituents was again shown in later experiments extending over a number of years (Gudernatsch and Olive Hoffman, 1929-36), when we studied the effects of amino acids, singly and in various mixtures, in tadpole development. Some of the simpler acids proved to be an adequate food for mere maintenance, some of higher molecular weight (arginine, lysine, cystine) supported growth, while the aromatic acids (phenylalanine, hydroxyphenylalanine, tryptophane) showed signs of a differentiation effect. All are alanine derivatives, the same as diiodotyrosine and thyroxine. In some experiments, iodine was added to the solution of these acids and the animals were reared in very dilute mixtures. The rate of development became more rapid in every case. In increasing degree of effectiveness, the best groups ranged as follows: tyrosine alone; phenylalanine+iodine; tryptophane+iodine; tyrosine+iodine. The next step toward a much greater and true hormonal effectiveness would be diiodotyrosine and thyroxine. Dr. Reineke's iodinated proteins would range at the true hormone end of such a graded series.

\* From: F. GUDERNATSCH. *Endocrine and Amino Acid Studies in the Physiology of Development* -- A Review of the author's experiments. 1936.

# THE BIOCHEMISTRY OF THE THYROTROPIC HORMONE

By A. ALBERT

*Endocrinology Laboratory, Section on Clinical Physiology, Mayo Clinic  
and Mayo Foundation, Rochester, Minnesota*

THIS paper will review the present status of the biochemistry of the thyrotropic hormone and present some recently published and some unpublished results as they are pertinent to the major topics of this field. After a few introductory concepts, the following major topics will be considered: bioassay, preparation and purification, thyrotropic hormone in body fluids, and chemical reactions. Since there are more than a thousand references to the thyrotropic or thyroid-stimulating hormone (TSH), there is no intent to prepare an exhaustive review. Four excellent recent reviews have considerably lightened this task; one covers the older literature, two deal with the chemistry of the hormones of the anterior lobe in general but contain sections on TSH, and one discusses the variations of TSH potency in the pituitary of various animals.<sup>1-4</sup>

## *General Concepts*

TSH may be defined as a substance obtained from pituitary tissue which, when given parenterally in proper dosage to various vertebrates, induces specific effects on the thyroid consisting of secretory alterations of the cytologic components of the follicular cells, hypertrophy and hyperplasia of the epithelium, vacuolization and resorption of colloid, loss of hormonal iodine, and increase of vascularity and of the size of the gland. As a result of such marked stimulation, there occur a number of secondary effects due to increased liberation of thyroid hormone, such as increased basal metabolism, loss of hepatic glycogen, acceleration of molting in urodeles, and acceleration of metamorphosis of amphibian larvae.<sup>5-32</sup> The possible extrathyroidal effects of TSH will not be discussed. The hormone is found only in the anterior of the three lobes of the pituitary and the evidence is overwhelming that a substance having identical or comparable properties is found in representatives of all vertebrate classes.<sup>4</sup> Whether the acidophiles or basophiles of the anterior pituitary form the thyrotropic hormone is an unsettled question, there being almost equal evidence for either cell type as its source.<sup>33-54</sup>

TSH is regarded as a separate pituitary hormone, distinct physiologically and chemically from all other known hormones of the pituitary gland. However, suggestions have been made that TSH is not the only hormone of the pituitary affecting the functional activity of the thyroid. Hypophyseal, thyroid-inhibiting substances have been postulated<sup>55-60</sup> but not confirmed.<sup>61</sup> Other workers have suggested two kinds of TSH,

one inducing histologic stimulation of the thyroid and another increasing the weight of the gland.<sup>62-64</sup> The existence of still different kinds of TSH has been suggested: one, a heat-stable hormone effective in amphibian metamorphosis and another, heat-labile, effective on the mammalian thyroid.<sup>9-11, 65</sup> Another interesting suggestion is that, depending on the animal source, TSH may exist as different proteins, having different specific activities and probably also different molecular weights.<sup>2, 3</sup> Finally, the relative species specificity of TSH (that is, the diminished efficiency of TSH prepared from the pituitary of one animal when tested on the thyroid of a distantly related animal) may also be construed as indicating different types of TSH.<sup>66-70</sup> For the present, TSH will be regarded as a single thyroid-stimulating hormone, leaving open the question of the number of hormones and chemical dimorphism. Although the evidence is not uniform, it is generally agreed that no gland other than the pituitary manufactures TSH.<sup>71 74</sup>

The content of thyrotropic hormone in terms of one or another type of unit representing activity per gram dry or fresh weight of either anterior lobes or whole pituitary has been assessed for various animals. Despite the discrepancies among the various units employed, the following list, not to be regarded with undue confidence, can be arranged reading from highest to lowest concentration of pituitary TSH: frog, sole, rat, mouse, dog, pig, sheep, toad, beef, turkey, man, horse, rabbit, cat, pigeon, chick, guinea pig, hen.<sup>4</sup> This order is of great interest in that there seems to be a direct relationship between the concentration of thyrotropin in the pituitary and the histologic appearance of the thyroid, namely, the more active the thyroid of an animal appears histologically, the greater will be the concentration of thyrotropic hormone in the pituitary of that animal.

If this general concept is valid, certain interesting corollaries have been and can be postulated. First, the sensitivity of the thyroid to exogenous TSH should be greatest in those forms possessing the smallest concentration of pituitary TSH, and therefore such animals should be superior forms for bioassay. The day-old chick and the guinea pig, which are among the lowest on the list, have indeed been considered excellent assay animals (at least among warm-blooded animals), while the forms having active thyroids and high thyrotropic hormone concentrations (rats) are refractory and therefore not as useful for this purpose. Another corollary may be that the rapidity of effect of the goitrogenic agents may be greater in those forms having a high concentration of thyrotropic hormone than in those having a lower concentration.<sup>75</sup> Some forms may actually be so lacking in thyrotropic hormone that goitrogens might be completely ineffective, as perhaps in certain axolotls and in the silver strain of dwarf mice. Possibly, the ability to trap iodine may be related to the TSH content of the pituitary. Data from the comparative physiology of the thyroid would be of value to test the correctness of these concepts.



The general relation between the pituitary and the thyroid is regarded as being reciprocal. Administration of thyroxine is followed by a reduction of the TSH content of the pituitary. On the other hand, administration of TSH induces a marked depletion of the thyroid hormone content of the thyroid. These are net effects, since the concentration of a hormone in a gland at any time represents the difference between the amount of hormone produced during a given time and the amount of hormone secreted during that same interval. As far as the pituitary is concerned, it is not known whether thyroxine induces a diuresis of TSH, a diminution of TSH production and secretion, or some other result. In the case of the thyroid, however, it is definitely known that administration of TSH causes a depletion of thyroid hormone by inducing a marked outpouring of hormonal iodine from the gland. Presumably, during this short interval, there is little to no effect on production rate. Other factors, such as sex, reproductive activity, season, diet, drugs, toxins and variations in external environment, which affect the relative amounts of thyrotropin, have been reviewed elsewhere.<sup>4</sup>

### *Bioassay of TSH*

Several of the current methods of assay of TSH are shown in TABLE 1.

TABLE 1  
SOME METHODS OF ASSAY OF TSH

<i>Reference</i>	<i>Test animal</i>	<i>End-point</i>	<i>Arbitrary sensitivity</i>
12-21	Guinea pig	Histologic	1
1, 77	Guinea pig	Histologic	1
95	Guinea pig	Weight	10
89	Guinea pig	Weight	4
96-98	Guinea pig	Cell height	< 1
99	Hypophysectomized rat	Metabolism	> 1
89	Chick	Weight	< 1
100	Chick	Iodine loss	0.1
85	Chick	Cell height	0.25
101	Tadpole	Metamorphosis	0.01
102	Guinea pig	Cytologic	0.001

When no data were given, I have estimated, however incorrectly, the relative sensitivity of the method, that is, the minimal amount of TSH necessary to produce a measurable effect. It is to be noted that the guinea pig and the chick were used more than any other animal, in accordance with the postulates made previously. In general, the plan of all these methods was to prepare serial dilutions of the substance to be tested, to inject them into animals one or more times a day for from one to six or more days, and then to determine one of the many end-points indicative of a thyrotropic effect. The least amount, or a multiple thereof, of the substance necessary to produce an effect has generally been regarded as a unit.

For various reasons, it is not possible to convert one unit accurately

to another. Too many modifications of each main method have been proposed and few of the proposals have been accompanied by quantitative data showing if, and how much, the proposed change affected the size of the unit to be supplanted. There is an astonishing lack of quantitative treatment of much of the data. Finally, although a standard such as the international standard is lacking, nonetheless a standard of one's own devising has rarely been employed concurrently with assays.

A few examples of the chaotic conditions will be given. In the guinea pig assay, for example, there is still controversy as to whether animals weighing 200 to 250 gm. give as reliable results as those of 100 to 150 gm.<sup>76-78</sup> Special diets,<sup>79</sup> special forms of pretreatment with various thyroid depressants,<sup>80</sup> and the use of special agents like colchicine to facilitate counting of mitoses<sup>81</sup> have been suggested in attempts to improve the bioassay. In the chick, the number of modifications of the method originally proposed by Smelser,<sup>82, 83</sup> utilizing thyroid weight of the day-old cockerel, is appreciable. There are now more than a half-dozen chick units.<sup>2, 84-92</sup> The specificity of the various kinds of end-points is debated. Furthermore, a number of authors have stated that the thyroid weight of the chick cannot be used as a measure of TSH since large thyroids may be encountered showing no histologic evidence of stimulation and, conversely, small thyroids may be found showing intense stimulation. The variations between strains of chicks and even within the same strain have been found to be enormous.<sup>93</sup>

Recent experience with the chick method has shown that the aperiodic variations, the effect of temperature, and the effect of season of the year are very large and that it was impossible to establish a fixed unit.<sup>94</sup> Nonetheless, the general method utilizing chicks can be modified so that it is satisfactory for certain types of investigation. For example, ten to twenty white Leghorn cockerels, one day old, were injected once daily for three days in each assay. The weight, mean cell height, and iodine concentration of the thyroid glands have been used as a measure of TSH potency. The relationship between dosage and effect can be seen by reference to curves published previously.<sup>94</sup> The exact values for these three end-points were not necessarily obtained at another time, when presumably the same dosages of TSH were given, but the type of dose-response curves was the same. This indicates that the response to apparently the same dosage of TSH is not constant or consistent, and, consequently, the so-called chick unit is a "freely floating" variable. The use of fixed units was deliberately avoided by the simple expedient of using a standard with each assay. The results of unknowns are given in terms of percentage deviation from the standard. These percentage deviations can then be compared with other deviations similarly calculated, but the exact values for thyroid weight, mean cell height, and iodine concentration cannot be so compared. It is of interest that the weight, iodine concentration, and mean cell height methods of evaluating TSH potency were in close agreement with one another. While in borderline

results a divergence may exist between weight and histologic end-points, which is partly understandable since the thresholds of these responses are different, no such divergence occurred when the dosage of TSH was sufficient to induce weights of thyroid between 4 and 9 mg.

It is not intended here to discuss the relative merits of each assay.<sup>1, 12-21, 77, 85, 89, 96-102</sup> From TABLE 1, it can be seen that the current methods are capable of detecting from about 0.1 to 10 arbitrary units or a range of a hundred-fold. The tadpole test and especially the cytologic test of De Robertis, although they have not yet been used widely enough, are promising since they represent methods useful for detection of the small amounts of TSH which may exist in blood and urine, and also of the high concentration of TSH in pituitary tissue.

In general, however, the current bioassay of TSH is unsatisfactory, especially if the various methods are compared with what one would regard as an ideal method. To rectify matters, an international standard of TSH (not of pituitary powder, which is available) should be established promptly. This should be a simple matter since TSH has been prepared in sufficient purity to serve as a standard. Although all difficulties may be solved by the use of this standard in conjunction with any of the current methods, there are, nevertheless, a certain number of attributes of which none of the current methods possesses all. These attributes are specificity, objectivity, sensitivity, convenience, and precision.

Specificity of action can be assured by the use of hypophysectomized animals. Objectivity can be achieved by the use of some specific action on the thyroid, which would be measured by an objective method. Histologic changes, unless they are striking, are not sufficiently objective, but a chemical determination of iodine concentration would be quite acceptable. Sensitivity can be achieved by appropriate choice of animal and end-point. The sensitivity should, if possible, be in the neighborhood of 0.001 arbitrary unit, so as to make the method widely adaptable. Convenience requires that the assay should be completed within a few hours, or at least during the same day, and not after a week of injections. Convenience also is to be considered in deciding on the other qualities of the assay. For example, the use of such an end-point as a determination of mean cell height can hardly be classified as a convenient method.

Although a few authors have treated their data<sup>89, 103</sup> by the usual mathematical methods applied to bioassay procedures, precision is the one factor which most methods thus far lack. The end-point to be used having been determined, it is a relatively simple matter to determine its variance in control animals. Having established a dose-effect relationship, one can determine how much of a difference in effect is safely above the normal variation of the controls. The minimal amount of hormone producing this effect is the minimal detectable dose. It is helpful to have a dose-effect range, however limited, in which the effect or a transformation of the actual response, such as the probit or logit method, is a linear or logarithmic function of dose. It is then possible to determine how small

a difference in dose can be detected by the method. It is also possible, then, to interpolate any unknown value in terms of the concurrently administered standard without having to waste time and animals balancing the known *versus* the unknown. The use of serial dilution, except to bring the dose within range of the sensitivity of the method, can thus be avoided. As a general principle, it may be safer to use as an end-point any of the primary effects of TSH, rather than any of the second-order effects mediated through the thyroid. These objectives would not be difficult to attain, and a great service to this field would be done by the establishment of a standard to be used in conjunction with a modern method of assay.

### *Methods of Preparation of Thyrotropic Hormone*

The methods used for preparation of thyrotropic hormone from either fresh or acetone-dried pituitary tissue will be reviewed here only in a general way, since White<sup>2,3</sup> has discussed this subject in considerable detail. The methods can be conveniently arranged in three groups.

In methods of the first group, TSH is extracted from pituitary tissue and the extract prepared as a dry powder. Junkmann and Schoeller<sup>1,77</sup> precipitated the initial alkaline pituitary extract with picric acid. After decomposition of the dye-protein complex, the active material was precipitated with acetone and dried. Rowlands and Parkes<sup>95</sup> precipitated TSH from alkaline extracts of the pituitary by means of alcohol. Such methods as these could be expected to lead to relatively little purification, since the reagents applied precipitate most proteins indiscriminately—the acid dyes by virtue of insoluble complexes with proteins and the organic solvents by virtue of protein insolubility, denaturation, or both.

More success attended the elimination of protein impurities of the extract before precipitation of the thyrotropic activity was carried out. Among methods of this second group, that of Loeser<sup>104-112</sup> can be cited. This author prepared alkaline extracts of pituitary tissue which were then treated with trichloroacetic acid. Large amounts of protein were thus precipitated, but some thyrotropic activity remained in solution. The active substance was then precipitated from solution by excess acetone (90 per cent). Bergman and co-workers<sup>113, 114</sup> used flavianic acid to precipitate TSH from solution after the initial extract had been cleared of bulky protein impurities by isoelectric precipitation. The same general plan with a few modifications was used by others,<sup>99, 115</sup> who precipitated an alkaline extract with calcium phosphate to remove certain non-thyrotropic protein. The supernatant solution was then saturated with ammonium sulfate, thus precipitating thyrotropic hormone which, after dialysis, was dried by the addition of alcohol. Another modification of this general plan was worked out by Lambie and Trikojus,<sup>116</sup> who used sulfosalicylic acid to precipitate large amounts of protein im-

purities from the initial extract and then precipitated TSH with sodium tungstate. After decomposition of the tungstate, the active material was adsorbed by benzoic acid, eluted with acetone, and dried. Preparations made by this plan have yielded greater activities than those of the first, since non-thyrotropic proteins were first removed from the extract, after which the extract was prepared in dry form by one procedure or another.

In the third group, the methods involve the use of fractional precipitation of the initial extract after it has been cleared of as much non-thyrotropic impurity as possible. Fraenkel-Conrat and co-workers<sup>117</sup> achieved highly potent preparations by making 0.25 per cent acetic acid-1 per cent saline extracts of beef pituitaries. This is a good initial extract, for it is equivalent to the cleared extract achieved previously in two steps. The thyrotropic hormone was precipitated from this extract by the addition of one volume of acetone. The precipitate containing TSH was then fractionated between 0.3 and 0.6 saturated ammonium sulfate and again between 39 per cent and 90 per cent acetone. Finally, the Yale group of investigators<sup>2, 118-121</sup> have described a process for the isolation of the hormone in an electrophoretically pure form. The initial extracts were made with 2 per cent sodium chloride at pH 7.6 and protein impurities were removed by isoelectric precipitation at pH 4.1 and by precipitation from a 50 per cent acetone solution. The active material was then precipitated by the further addition of acetone to 75 per cent and the precipitate after solution in water was fractionated by lead acetate and trichloroacetic acid. The acid solution was dialyzed and lyophilized. The resulting protein was found to be a pure protein by sedimentation and electrophoretic data. The molecular weight was calculated to be about 10,000 and the activity of the preparation was stated to be one chick unit per microgram.

Attempts to use physical methods of purification, such as ultracentrifuging crude extracts or partly purified preparations, were quite unsuccessful.<sup>122, 123</sup> The use of a wide variety of adsorbents<sup>92</sup> was only partly successful in certain instances and, consequently, further attempts utilizing this technique have been abandoned.

A comparison of these methods and the yields obtained are shown in TABLE 2.<sup>77, 95, 113, 116-118</sup> Some of the authors have not given sufficient data with which such tabulation could be arranged, and so a few personal guesses and averages were made. Of the six examples shown, five different methods of assay have been used. Since the units are different and not readily convertible, the only method of comparison of the final product obtained in each case is that of the degree of purification achieved over the starting source. This value can then be compared with similar values obtained by other workers, but the activity of the final product cannot be so compared. The methods of the first group give good yields but poor purification, since the general procedure amounts to no more than a drying of the initial extract of the pituitary. In the second group, in which impurities were first removed from the extract, after which the

TABLE 2  
COMPARISON OF SOME METHODS OF PREPARATION OF TSH FROM BEEF PITUITARY

Reference	Start		Finish			Comparison			
	Solids, kg.	Units, $\times 10^3$	Units, per gm.	Solids, gm.	Units, $\times 10^3$	Units, per gm.	Yield solids, per cent	Yield units, per cent	Purification
95	0.1	3 (?)	30	10	2.5	250	10	80	8X
77	1.0	375	375	20	400	20,000	2	100	50X
116	0.1	37.5 (?)	375	1.4	14	10,000	1.4	38	25X
113	0.66	33	50	7.8	31	4,000	1.1	90	80X
117	1.0	660	660	2.5	173	67,000	0.25	26	100X
118	0.2	625 (?)	3,100	0.5	500	1,000,000	0.25	80	320X

extract containing TSH was prepared in dry state, higher purification results. Fractional precipitation, characterizing the third group of methods, has yielded the highest purification. Ordinarily, great losses attend the use of fractional precipitation methods. Fraenkel-Conrat and co-workers have reported a loss of some 74 per cent of activity during manipulation, whereas Ciereszko<sup>118</sup> has apparently retained 80 per cent of the initial activity.

The efforts in this field have resulted in the isolation of an amorphous protein claimed to be pure TSH.<sup>2</sup> There are two types of criteria employed to assess the purity of a pituitary protein. The first type is physiological, *i.e.*, the hormone in question must not possess any physiological action attributed to other hormones of the pituitary as determined by appropriate biological tests. The second type is a physico-chemical proof, commonly applied to proteins in general. The first criterion has not been met. Fraenkel-Conrat and co-workers, who are singular in that they have reported in excellent detail the physiological properties of their product, stated that it was contaminated with prolactin to the extent of 0.025 per cent, adrenotropic hormone 3 per cent, growth hormone 1 per cent, FSH 0.4 per cent, and LH 10 per cent. Ciereszko<sup>118</sup> stated that 5 mg. of his amorphous protein did not give a reaction indicative of prolactin. He also stated that the product contained neither growth hormone nor gonadotropic hormone, but the doses used in performing these tests were not stated. If 1 mg. of a preparation gives no gonadotropic effect, it is not certain that 5 mg. might not give a positive reaction. In the absence of details as to dosage and of tests for other pituitary hormones, it would appear that the purity of the TSH isolated has not yet been proved by a sufficient number of the physiologic criteria. White<sup>2</sup> stated that a similar TSH protein prepared from sheep pituitary is twice as active as that prepared from beef (1 chick unit per 0.5 microgram), but that the product from sheep is contaminated with gonadotropic hormone. It is possible that the beef TSH protein could be pure and be simultaneously less than half as active as the same hormone prepared from sheep, a situation which would indicate, if true, that thyrotropin could exist as different proteins.

The chemical and physical criteria of purity are not within the scope of this discussion. Homogeneity in the ultracentrifuge or in the electrophoresis apparatus is part of the proof of purity. It is to be recalled that constant specific activity, crystallization, constant specific activity on repeated crystallization, and solubility data are other criteria to be applied. Data of this kind for TSH do not as yet exist.

No purified preparation of TSH comparable to those described is readily available for the thyroid physiologist, who must, by necessity, use relatively impure material. Since an impure TSH preparation is generally available (Antuitrin T, Parke, Davis & Company), and since most investigators in this country have been using it for the last ten years, it was thought worth while to explore the possibilities of improv-

ing its purity. Antuitrin T is available as a sterile solution in vials of 10 ml. each, 1 ml. containing 50 Junkmann-Schoeller units and 20 mg. of protein. The following method, which is simple enough to be carried out by technical aid, was worked out.<sup>124</sup>

The contents of a 10-ml. vial of thyrotropic extract are transferred to a 50-ml. centrifuge tube. Distilled water (10 ml.) is added to wash the vial and then is similarly transferred to the centrifuge tube. One volume (20 ml.) of 10 per cent trichloroacetic acid is added to the centrifuge tube, the contents are mixed, and the tube is placed in a refrigerator for one hour at 5° C. A heavy, white precipitate is formed which is centrifuged off. The supernatant solution is stored and the precipitate is emulsified in 10 ml. of water. A few drops of 0.1 N NaOH are added until solution is complete. Trichloroacetic acid (10 ml.) is again added and the tube is placed in the refrigerator for one hour. The heavy, white precipitate is centrifuged off and discarded and the combined supernatant fluid is adjusted to pH 7.0. Two volumes of saturated ammonium sulfate solution are added and the mixture is allowed to stand in the refrigerator overnight. The white precipitate is either filtered or centrifuged off, and the solution is discarded. The precipitate is dissolved in 10 ml. of water and 5 ml. of saturated ammonium sulfate solution is added. After standing from one to two hours in the refrigerator, a brownish precipitate is formed which is removed and discarded. To the supernatant solution is added 5 ml. of saturated ammonium sulfate solution and after standing overnight the precipitate is centrifuged off, dissolved in 5 ml. of water, and dialyzed against distilled water until salt-free. To the dialyzed solution is added M metaphosphoric acid to pH 3.0. The precipitate containing thyrotropic hormone which forms immediately is centrifuged off. The best preparations had an activity of 40 Junkmann-Schoeller units per mg. Eight milligrams of the preparation had no gonadotropic activity, which is present to an appreciable extent in Antuitrin T, owing to the separation and removal of FSH and LH during the procedure. When Ciereszko's method appeared, it was of interest to work up some Antuitrin T according to his procedure. Starting with the acetone precipitation step at pH 4.1, no increase in purity beyond 40 units/mg. was obtained. However, the failure to achieve a more potent product may have been due to differences in extraction and preparation of Antuitrin T.

### *TSH in Body Fluids*

This aspect of the biochemistry of TSH is in a very confused state owing to the unsatisfactory nature of the bioassay and to the difficulties inherent in the protein nature of TSH. Since most current methods vary in sensitivity from 0.1 to 10 arbitrary units, they are not sufficiently delicate to detect minute amounts of hormone that may exist in body fluids, unless chemical methods are used to concentrate the material. For example, human blood serum may contain 1 arbitrary unit per 200



ml. of serum, an inference based on certain observations in the literature and some personal experience with human plasma. It would be impractical to withdraw the corresponding amount of blood from patients with thyroid disorders and, furthermore, this amount would suffice for only one test animal, provided the animal could tolerate it. A more realistic amount of blood to be withdrawn is 10 ml., which would contain, on the foregoing basis, a total of about 0.02 arbitrary unit, an amount of hormone which current methods cannot detect unless the serum is concentrated.

Similar difficulties apply to urine. If one considers only the positive results in the literature, one would estimate the TSH content to be about one unit per 200 ml. of urine. There is, of course, no problem in the amount of urine to be obtained, but it is not possible to inject such amounts unless the urine is concentrated by chemical or physical means. The common bond in this respect between blood and urine is that both may contain only traces of TSH. Otherwise, the chemical approach to the fractionation of blood and urine is different and will be described separately.

Tests on untreated blood serum (TABLE 3)<sup>99, 124-146</sup> in amounts up to

TABLE 3  
SUMMARY OF SOME TESTS FOR TSH IN BLOOD SERUM (NO TREATMENT OF SERUM)

Reference	Source of serum	Volume per test animal, ml.	Assay method	Results
124-144	Rat, rabbit, dog	15	Guinea pig, histologic	+
	Guinea pig	15	Guinea pig, histologic	0
	Human (n., thy., myx.)	15	Guinea pig, histologic	+
	Human (obesity)	15	Guinea pig, histologic	0
	Human (acr., a.p. trauma)	15	Guinea pig, histologic	+
	Rat	40	Rabbit, histologic	+
	Human	40	Rabbit, histologic	+
145	Human (n.)	15	Guinea pig, histologic	0
	Human (thy.)	15	Guinea pig, histologic	+
	Human (acr.)	15	Guinea pig, histologic	0
	Dog (hyp.)	15	Guinea pig, histologic	+
	Dog (n., thyroidectomy)	15	Guinea pig, histologic	0
146	Human (thy.)	10	Hyp. rat, histologic	0
	Human (myx.)	10	Hyp. rat, histologic	+
99	Human (Simmonds')	?	Guinea pig, B.M.R.	Less than normal

Abbreviations: n. = normal; thy. = thyrotoxicosis; myx. = myxedema; acr. = acromegaly; a.p. = anterior pituitary; hyp. = hypophysectomized; Simmonds' = Simmonds' disease.

40 ml. per animal have yielded widely divergent results, and it cannot be stated that TSH has been unequivocally found in serum. The fractionation of serum for TSH has also yielded unsatisfactory results (TABLE 4).<sup>147-151</sup> Blood represents a concentrated solution of protein, and the problem is to remove as much non-TSH protein from the serum as possible while keeping TSH in solution, after which the protein fraction containing TSH can be precipitated. Remembering the adsorptive prop-

TABLE 4  
SUMMARY OF SOME TESTS FOR TSH IN BLOOD  
SERUM (WITH CHEMICAL TREATMENT OF SERUM)

Reference	Source	Treatment	Volume per test animal, ml.	Method of assay	Results
147-148	Rabbit, human (n.)*	Acetone ppt.	10	Guinea pig, weight	0
	Human (thy.)	Acetone ppt.	10	Guinea pig, weight	0
	Human (myx.)	Acetone ppt.	10	Guinea pig, weight	0
	Rabbit (thyroidectomy)	Acetone ppt.	10	Guinea pig, weight	+
149-151	Human (n., thy., myx.)	Acetone ppt.	10	Guinea pig, histologic	+

\* For explanation of abbreviations used previously see TABLE 3. Further abbreviations: ppt. - precipitate.

erties of bulky protein precipitates, the loss of TSH entailed in such procedures would be great and would tend to nullify the gains obtained by concentration. The reverse procedure of precipitating TSH selectively from the bulk of serum proteins seems impossible in view of the unselective nature of the precipitating reagents. For the fractionation of blood, advantage has been taken of the solubility of TSH in 40 to 50 per cent alcohol or acetone. The serum proteins insoluble in this concentration of reagent are removed, after which the proteins remaining in solution are precipitated by the further addition of reagent to 80-90 per cent. The results (shown in TABLE 4) obtained by use of this method again permit no definite conclusions regarding the presence or absence of TSH in serum.

A number of procedures for recovering TSH in blood, including the reported methods, have been studied.<sup>152</sup> For example, when 500 Junkmann-Schoeller units of pituitary TSH (Antuitrin T) were added to 500 ml. of whole blood, it was found that about 10 per cent of the TSH remained with the erythrocytes, probably by adsorption. About 20 per cent was found in the 40 per cent acetone precipitate, and 40 per cent of the activity seemed to have disappeared completely, leaving about 30 per cent actually recovered. The disappearance is puzzling but has been noted when other hormones were added to fresh serum and the mixture was immediately assayed. Other methods of extraction and concentration of TSH were also found to recover part of the activity. In any case, it seems certain that various methods are capable of recovering part of pituitary TSH added to blood, but the same methods applied to clinical material have not yielded indisputable evidence that TSH is actually present in blood.

The tests performed with urine are equally divergent. Amounts up to 50 ml. of untreated urine have been tested and it cannot be stated that TSH has been definitely demonstrated (TABLE 5).<sup>124-144, 146, 153-160, 164, 165</sup> The bulk of the work, however, has been done by concentrating human urine. Urine, unlike blood, is a very dilute solution of protein, and the problem is to remove the protein quantitatively from a large amount of solution. The losses of TSH thus entailed are equally large but are differ-

TABLE 5

## SUMMARY OF SOME TESTS FOR TSH IN URINE (NO TREATMENT OF URINE)

<i>Reference</i>	<i>Source of urine</i>	<i>Volume per test animal, ml.</i>	<i>Assay method</i>	<i>Results</i>
124-144	{ Rat, dog, rabbit	15	Guinea pig, histologic	+
	{ Guinea pig	15	Guinea pig, histologic	0
	{ Human (n., thy., myx.)*	15	Guinea pig, histologic	+
	{ Human (pregnant)	40	Rabbit, histologic	0
153	Human (acr., Cushing's, n.)	15	Rabbit, histologic	+
154	Human (acr.)	15	Rabbit, histologic	+
155	Human (thy., p.o. exoph.)	30	Guinea pig, histologic	0
156	Human (n.)	16	Rabbit, histologic	+
146	{ Human (thy.)	50	Hyp. rat, histologic	0
	{ Human (myx.)	50	Hyp. rat, histologic	+
157-160	Human (thy.)	39	Guinea pig, histologic	0
164	Human	16	Guinea pig, histologic	0
165	{ Human (n.)	16	Rabbit, histologic	+(33%)
	{ Human (various disorders)	16	Rabbit, histologic	+(50%)

\* For explanation of abbreviations used previously see TABLE 3. Further abbreviations: Cushing's - Cushing's syndrome; p.o. exoph. = postoperative exophthalmos.

ent from those attending the fractionation of serum, for they are due to incomplete precipitation, losses on glassware, and so forth. The methods used to recover TSH in urine have all revolved about the use of protein precipitants such as organic solvents, alkaloidal reagents, or adsorbents. Amounts of concentrate representing 50 ml. to 6 liters of urine have been tested for TSH (TABLE 6).<sup>90, 97, 147, 148, 157-163</sup> Again, one is not cer-

TABLE 6

SUMMARY OF SOME TESTS FOR TSH IN HUMAN URINE  
(WITH CHEMICAL TREATMENT OF URINE)

<i>Reference</i>	<i>Source</i>	<i>Treatment</i>	<i>Volume per test animal, liters</i>	<i>Assay method</i>	<i>Results</i>
161	Pregnant	Tungstate ppt.	?	Guinea pig (?)	+
97	{ N., thy.,* thyroidectomy	Acetone ppt.	0.05	Guinea pig, cytologic	+
	Myx.	?	?	?	0
162	{ Myx.	Acetone ppt.	0.2-0.4	Chick, cytologic	0 (1 case +)
	{ N., acr., Cushing's	Acetone ppt.	0.1	Chick, cytologic	0
	{ N., cretin, myx.	Alcohol ppt.	0.2-0.5	Guinea pig, histologic	0
163	{ Thy.	Alcohol ppt.	0.2-0.5	Guinea pig, histologic	0
	{ P.o. thy.	Alcohol ppt.	0.2-0.5	Guinea pig, histologic	+(transient)
157-160	Thy.	Alcohol ppt.	2-6	Guinea pig, histologic, and B.M.R.	0
147-148	{ N.	Benzoic ads.	0.1-2.5	Guinea pig, histologic	0
	{ Thy.	Benzoic ads.	0.1-2.5	Guinea pig, histologic	12% of cases +
	{ Myx.	Benzoic ads.	0.1-2.5	Guinea pig, histologic	0

\* For explanation of abbreviations used previously, see TABLE 3. Further abbreviations: ads. = adsorbate; p.o. thy. = postoperative thyroidectomy.

tain that this hormone has or has not been demonstrated as a constituent of urine.

The question always arises as to how completely such methods actually recover thyrotropic hormone, a question that can be answered at present only by adding pituitary TSH to urine and working up the artificial mixture. The recovery of TSH by all of the previously reported methods and by a few new ones has been studied.<sup>152</sup> Briefly, rich artificially prepared mixtures of urine and TSH, containing 1 unit per 30 ml. of urine, can be concentrated with recoveries of about 50 to 90 per cent. However, when poor artificially prepared mixtures containing 1 unit of TSH per 300 ml. of urine were used, only 20 to 50 per cent of the added TSH could be recovered. In general, the same impasse reached with blood has been attained in studies of urine—pituitary TSH can be recovered with varying success when it is added to urine, yet the application of the same methods to urine of patients has, on the whole, yielded conflicting results. There is also a contradiction in the literature, for positive reactions for TSH have been obtained with concentrates equivalent to 50 ml. of urine, and it is to be anticipated that ten times as much concentrate would induce a profoundly stimulating effect on the thyroid, a situation which has not often been reported.

It seems impossible to reconcile such conflicting data, but a few possible explanations can be considered and a few suggestions can be made. One factor may be that antithyrotropic substances of thyroid and extra-thyroid origin may have been concentrated simultaneously with TSH, and thus the TSH effect of the concentrate would be nullified.<sup>166</sup> Another factor may be the unspecificity of the assay method. A careful analytical study has led to the conclusion that some impure pituitary TSH preparations induce histological signs of intense stimulation without increasing thyroid weight in the guinea pig, whereas this was not encountered with purified TSH.<sup>103</sup> Since concentrates from blood and urine are less pure than even the crudest pituitary extracts, it follows that these dichotomous effects constitute a danger in all studies utilizing histological or cytological methods of assay. A third consideration is that an assumption has been made that TSH in body fluids is the same substance as pituitary TSH, and that it can be recovered by methods which recover pituitary TSH when added to such fluids as a tracer. This is a dangerous assumption and one unsupported by any critical evidence.

In view of these considerations, it would be well to subject this field to a reinvestigation utilizing a bioassay delicate enough to detect from 0.01–0.001 arbitrary units of TSH. TSH action in untreated blood and urine having been demonstrated, the fractionation of these fluids can then proceed on the same basis as for the fractionation of any unknown substance for which a biological test exists. Only when this is done will it be possible to determine whether the thyrotropic hormone in body fluids resembles the hormone as obtained from the pituitary in its chemical or physical properties, or whether it circulates in the form of a new

compound due to its metabolism or to its transport in blood. More important still is that, by simply increasing the dose of the purified concentrate, it will be possible to determine whether or not the concentrate possesses all of the physiological properties of pituitary TSH enumerated earlier. So far, it has been assumed that any one of the end-points (usually histological) constitutes *bona fide* evidence for the presence of TSH and, consequently, no one has tested the concentrate for all of the important actions of TSH. It is to be regretted that this precaution of conclusively demonstrating the physiological identity of urinary or blood concentrate with pituitary TSH seems to have been partly neglected.

### *Reactions of Thyrotropic Hormone*

Most of the chemical reactions of TSH that have been reported are reactions of the hormone in impure state and will have to be repeated when enough pure material becomes available. The only basis used for evaluating these reactions is the loss or preservation of biological activity; certainly, chemical formulation in terms of composition or active groups is not possible.

In general, the reactions of TSH as a protein are not remarkable. Except for two divergent reports,<sup>9, 11, 65</sup> it is agreed that the biological activity is lost on boiling.<sup>2</sup> The divergent opinion may result from the fact that the effect of temperature on the biological activity of a protein depends, among other things, on the presence or absence of ions, the pH of the solution, and the presence or absence of impurities. TSH activity is destroyed by proteolytic enzymes such as pepsin, trypsin, and chymotrypsin, but not by papain and carboxypeptidase.<sup>167</sup> TSH is precipitated by several reagents which precipitate proteins, such as picric, picrolonic, tannic, phosphotungstic and flavianic acids, and by such salts as Reincke's salt, mercuric chloride, gold chloride, silver nitrate, and uranium acetate. It is not precipitated by sulfosalicylic acid or by trichloroacetic acid from purified solutions, but 60 to 90 per cent of the thyrotropic activity is carried with the bulky precipitate, presumably by adsorption, when these acids are used on impure pituitary extracts.<sup>92</sup> TSH is also readily adsorbed by a variety of materials, such as colloidal iron hydroxide, carbon, fuller's earth, permutit, ion exchangers, and benzoic acid.<sup>1, 92, 168</sup>

The solubility of TSH in salt solution such as ammonium sulfate and sodium sulfate leads one to the conclusion that its behavior is that of the so-called pseudoglobulin type of protein—being soluble in water and in dilute salt solutions but insoluble in more concentrated salt solutions (0.5 saturated ammonium sulfate solution). It is soluble in dilute solutions of organic solvents miscible with water such as 30 to 40 per cent alcohol or acetone, but becomes progressively insoluble in more concentrated solutions such as 60 to 80 per cent acetone. Advantage of this property has been taken in the purification of TSH. It will be recalled

that TSH was precipitated from saline-acid extracts by adding acetone to 50 per cent by volume.<sup>117</sup> However, others<sup>118</sup> using essentially the same procedure found that TSH remained in the 50 per cent acetone solution. Like the effect of boiling, the solubility of TSH is dependent on temperature, pH, concentration of inorganic ions and other factors, and it is not surprising that even slight changes of conditions may profoundly influence the solubility of TSH in 50 per cent acetone.

TSH does not pass cellophane membranes. Because it is soluble in water throughout a rather wide range of hydrogen ion concentration, it was stated<sup>99</sup> that TSH has no isoelectric point. A direct determination of its mobility in an electric field has not been made, however, and it is to be recalled that this is the true basis for determination of an isoelectric point. TSH gives certain of the color reactions of proteins. Its elementary composition is not characteristic, *viz.*, 12 to 13 per cent nitrogen, 5 to 6 per cent hydrogen, 42 per cent carbon.<sup>2</sup> A content of 2.5 per cent of glucosamine has been reported.<sup>117</sup> Acetylation, benzoylation, and reduction by cysteine under certain conditions resulted in loss of thyrotropic activity.<sup>117</sup>

In addition to these reactions, the behavior of TSH to a variety of simple substances has been studied.<sup>94, 152</sup> As an example of the plan of these experiments, 6 mg. TSH (Antuitrin T) was added to a series of tubes containing 10 ml. of various oxidizing agents. The tubes were incubated for one hour at 37.5° C. and then diluted with distilled water to 45 ml. No attempt was made to buffer the system. One milliliter was injected subcutaneously daily for three days in a group of fifteen chicks, each chick thus receiving a total of 0.4 mg. or about 1 unit of TSH, a dosage of hormone which lies in a very sensitive portion of the dose-response curve. The results of the assay as determined by thyroid weight, mean cell height, and iodine concentration were expressed as percentages of the control standard.

The effect of certain oxidizing agents on thyrotropic potency is shown in TABLE 7. Permanganate and iodine induced complete inactivation of thyrotropic effect and were studied more extensively than the other

TABLE 7  
EFFECT OF CERTAIN OXIDIZING AGENTS ON TSH\*

<i>Treatment</i>	<i>Inactivation, per cent</i>
0	0
0.01 M $K_3Fe(CN)_6$	50
0.01 M $K_4Fe(CN)_6$	40
0.01 M $CrCl_3$	50
0.01 M $K_2Cr_2O_7$	35
0.01 M $KMnO_4$	100
0.01 M $Ba(MnO_4)_2$	100
0.01 M $H_2O_2$	20
0.01 M $Na_2SeO_4$	20
0.01 M $I_2$ in KI	100

\* 0.4 mg. of TSH per chick.

agents. The effect of various concentrations of permanganate on TSH activity (TABLE 8) indicated that the degree of inactivation was propor-

TABLE 8  
EFFECT OF  $\text{KMnO}_4$  ON TSH ACTIVITY

<i>TSH per chick, mg.</i>	<i>Concentration of <math>\text{KMnO}_4</math>, molarity</i>	<i>Inactivation, per cent</i>
0	0	
0.8	0	0
0.8	0.1	100
0.8	0.01	100
0.8	0.001	76
0.8	0.0001	46
0.8	0.00001	0

tional to the concentration of the reagent.<sup>152</sup> Simultaneous assay showed that permanganate abolished the net gonadotropic potency as well as the thyrotropic activity as determined by the weights of the testes and thyroid of the same test animal (TABLE 9). On the other hand, iodine seemed

TABLE 9  
SIMULTANEOUS EFFECT OF  $\text{KMnO}_4$  ON THYROTROPIC AND GONADOTROPIC  
POTENCY OF TSH EXTRACT

<i>Extract per chick, mg.</i>	<i>Treatment</i>	<i>Average thyroid weight, mg.</i>	<i>Average testes weight, mg.</i>
0	0	4.0	7.5
0.8	0	7.7	16.9
0.8	0.01 M $\text{KMnO}_4$	4.0	6.5
0	0.01 M $\text{KMnO}_4$	4.0	7.4

to have a more selective action on TSH than on gonadotropins (TABLE 10). Although not strictly comparable with the permanganate experi-

TABLE 10  
SIMULTANEOUS EFFECT OF IODINE ON THYROTROPIC AND GONADOTROPIC ACTIVITY

<i>Extract per chick, mg.</i>	<i>Treatment</i>	<i>Average thyroid weight, mg.</i>	<i>Average testes weight, mg.</i>
0	0	3.19	9.20
0.8	0	6.60	16.40
0.8	Iodinated	3.25	12.00
8.0	0	9.60	26.30
8.0	Iodinated	4.90	26.50

ment, this experiment demonstrated that the TSH activity of Antuitrin T was reduced to the extent of 90 to 100 per cent, whereas the gonadotropic potency of the extract was not seriously affected.

The reaction of TSH and iodine merited further study in view of the well-known antithyrotropic activity of iodine on the thyroid and of its central role in the biosynthesis of the thyroid hormone. If a small amount of TSH extract (20 mg.) was mixed with 10 ml. of Lugol's solution and left at room temperature for half an hour to two hours, a brown precipi-

tate appeared which was removed by centrifuging and washed with water. This precipitate, when properly diluted for assay, did not yield any thyrotropic effect; yet it represented most of the protein of the extract that was present in the mixture. Moreover, the activity was not present in the supernatant fluid or the washings. That the TSII was actually in the precipitate in an inactivated form will be made manifest later, since it was possible to treat this precipitate in many ways to recover the original potency. The reaction between iodine and the extract occurred in an acid medium (pH 5.0) and yielded a mixture of insoluble iodinated proteins, containing 10 per cent iodine. Part of the iodine (40 per cent) seemed tightly bound to the proteins, possibly absorbed, whereas 60 per cent was very loosely bound, perhaps adsorbed. It could be determined that the tightly bound iodine was not responsible for the loss of TSII activity, but that this was due to the loosely incorporated iodine.<sup>94</sup>

The presence of iodine in the inactivated TSII made it imperative to determine whether the inactivation was due merely to the biological action of iodine as such on the thyroid, or whether it represented a chemical alteration of the active protein of the extract. The first experiment consisted in adding variable amounts of iodine to a constant amount of TSH and comparing the potency of the mixture with the standard. It was found that the inactivation of TSII was dependent on the amount of iodine added. It was shown, furthermore, that no inactivation occurred unless the system contained free iodine. The minimal amount of iodine inducing maximal inactivation was calculated to be 100 micrograms of iodine per unit of TSH. This calculation was in fairly good agreement with the actual determination of free iodine in the iodinated precipitate (24 micrograms of iodine per unit of TSII). The difference of some 75 micrograms probably represented the excess free iodine in solution.

It was of interest to determine whether the inactivation of TSII was independent of the amount of iodinated TSH tested. In these experiments, variable amounts of TSH were added to an excess of Lugol's solution. The precipitates were removed and emulsified in water for assay. Whether 2 or 20 original units of TSH were given to each animal, the proportion of inactivation remained fairly constant, between 95 and 100 per cent.

That the inactivation of TSH was not due to the biological action of the iodine contained in the precipitate was determined by comparing mixtures of TSH with iodine as iodide, as molecular iodine in iodide, and as molecular iodine dissolved in alcohol, in various doses of from 10 to 10,000 micrograms. The results showed that iodide did not abolish the effect of TSH nor did it form insoluble precipitates of TSH, whereas free iodine in iodide or free iodine alone did. The effect of each form of iodine alone in varying amounts was of course nil, except to increase the thyroid iodine concentration slightly.

It was, therefore, apparent that the inactivation of TSH by iodine



was not due to the biological action of iodine on the thyroid of the test animals. That it was not the result of any thyroxine forming in the reaction was indicated by mixing synthetic thyroxine in amounts exceeding the theoretical maximal yield of thyroxine that could have been produced by iodination of protein of the extract with TSH and assaying the mixture. Such a mixture resulted in only slight depression of TSH activity and could, therefore, not account for the complete loss observed. It was, furthermore, improbable that the loss of TSH activity could be attributed to an alteration of tissue absorption of the iodinated product, as, for example, some change induced by an irritating action of the free iodine. Furthermore, when the iodinated protein was dialyzed against large quantities of water, much of the excess free iodine was removed, and yet the dialyzed material remained completely inactive.<sup>152</sup>

The inactive iodinated material can be restored to activity by a variety of measures.<sup>169</sup> In these experiments, TSH was accurately added to a constant excess of Lugol's solution and the resulting precipitates were removed. After emulsification, the precipitates were triturated with various reducing agents in amounts just necessary to cause decoloration of the free iodine. In the first series studied, the reducing agents were 2-thiouracil, KSCN,  $\text{Na}_2\text{S}_2\text{O}_3$ , 2-aminothiazole, 6N propyl thiouracil, 5-amino-2-mercaptothiadiazole, ascorbic acid, and 3-phenylaminoethyl-2-mercaptothiazoline. When decoloration of iodine occurred, the precipitate immediately dissolved, yielding a water-clear solution much like the original Antuitrin T. The clear solution contained the original TSH activity that was added. Assuming that 5 per cent of the original activity remained in the inactivated precipitate (actually, none could be demonstrated), the thyrotropic potency of the iodinated product after reduction, as compared with the standard, was between 1,000 and 2,000 per cent greater than before reduction. A second series of substances consisting of benzyl thiouracil, cysteine, glutathione, tetramethylthiouracil, 2-5 dithiothiadiazole, 2-thiobarbituric acid, and thiobarbital was also found to restore the activity of the iodinated complex. The extent of the reactivation, like that of the first series, was between 1,000 and 2,000 per cent as compared with the controls. In brief, these experiments showed that the restoration of activity was related to the reducing power of these agents, but not necessarily to their goitrogenic ability.

Since this reaction system contained three physiologically active reactants, it was essential to test them separately in all possible combinations. Thus, the same amount of reducing agents of the first series, when given alone, had no effect on the thyroid, nor did the same amount of iodine (200 micrograms) as contained in the iodinated precipitate when mixed with the reducing compounds induce any effect on the thyroid. The effect of iodine alone, of iodide mixed with the TSH, and of TSH alone has been described. From these control experiments, the conclusion was drawn that the restoration of TSH activity was due to an interaction of the iodinated complex and the reducing substances.

Since it might be thought that the reactivation resulted from some augmenting action of the reducing compounds on the assumed small portion of TSH escaping inactivation during iodine treatment, another type of experiment was done by mixing active TSH with these reducing substances in the same dosage. The results indicated that the biological potency of thyrotropic extract was enhanced over that of the control standard.<sup>170</sup> Furthermore, such an augmentation was present even after the reducing compounds had presumably been removed from the extract prior to bioassay by dialysis or after the hormonal material had presumably been removed from the agents by precipitation with ammonium sulfate. When the percentage augmentation was calculated per milligram of augmenting reagent, it was seen, in the first series, that goitrogens were far more effective than nongoitrogenic agents. Indeed, ascorbic acid induced a marked destruction of thyrotropic hormone, rather than augmentation.

The second series of agents was studied in a preliminary fashion for their augmenting ability.<sup>152</sup> Augmentation was induced by benzyl thiouracil, cysteine, tetramethylthiourea, 2-5 dithiothiadiazole, 2-thiobarbituric acid, and thiobarbital. However, cysteine did not affect TSH, and glutathione, like ascorbic acid, caused a decrease of TSH potency. Sufficient data were not available to determine whether in the second series there was still a correlation between the amount of augmentation and the goitrogenic potency of the various compounds.

The interactions of TSH, iodine, and goitrogens are of interest since all of these substances are in themselves physiologically active. Moreover, some of these effects have been duplicated *in vivo*.<sup>171</sup> It seems impossible, however, to attempt an explanation for them on chemical grounds. The TSH used in these experiments was quite impure, and it is not known that augmentation resulted from some alteration in the TSH molecule, or that it actually resulted from destruction of an inhibiting material present in Antuitrin T, or that some inactive material was converted. The effect of iodine may be thought of as an oxidation of proteins of the extract, including perhaps the protein representing TSH. The reactivation would seem to be due simply to reduction of the free iodine. The augmenting effect of reducing compounds might be related to their reducing potency, but this does not explain the reverse effects of ascorbic acid and glutathione. Possibly, the reducing potential is more important in this respect than the total reduction. Since the thyrotropic hormone represented less than 1 per cent of the total protein of the extract, it would be unwise to hazard a guess that these substances actually reacted with the TSH protein. Consequently, further exploration along these lines will be fruitless until such experiments can be repeated with pure thyrotropic protein, for only then will it be possible to obtain some definite clue as to the nature of the changes induced in TSH potency.

Aside from the physiological interest in such a study, it is of interest

to learn what determines the biological activity of certain protein molecules. TSH has no prosthetic group, as far as can be determined, and so the biological activity may depend on the presence of certain chemical groups, or arrangement of these groups, or to some orientation of the groups to the molecule as a whole. Although many groups may be essential for physiological activity, it is possible that changes in any one group may lead to destruction of biological activity. It may be possible that some groups are more important than others in this respect. Consequently, acetylation of a protein, or changes in the S-S-bonds by thiol compounds, or substitution of iodine in the aromatic residues, may all seriously affect its biological activity, but it does not necessarily follow that one or another of these groups is responsible, *per se*, for the activity. To ascribe, as has been done, the physiological action of TSH to one or another group or, vaguely, to its "protein nature" is equally unsatisfying, but nothing better can be done until the chemistry of physiologically active proteins has been appreciably advanced.

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# PHYSIOLOGICAL REACTIONS OF THE THYROID-STIMULATING HORMONE

By RULON W. RAWSON

*Thyroid Clinic, Massachusetts General Hospital, and Department of Medicine,  
Harvard Medical School, Boston, Massachusetts\**

A VARIETY of thyroidal and extrathyroidal changes have been attributed to actions of the thyroid-stimulating hormone of the anterior pituitary. It is not the purpose of this paper to review the extensive literature concerning these varied effects observed in animals treated with pituitary extracts rich in thyroid-stimulating substances. Indeed, this discussion will be more or less limited to studies, done in our own laboratory, which we think tend to throw some light on the primary effects and mode of action of this hormone of the pituitary.

Some of the recent studies in which radioactive iodine has been used would indicate that the thyrotrophic hormone is related to the thyroid's metabolism of iodine. These studies merit consideration and discussion. Hertz and associates,<sup>1</sup> in some of the earlier studies with radioactive iodine, observed a parallel increase in the basal metabolic rate, thyroid hypertrophy, and collection of radioactive iodine in rabbits treated with thyroid-stimulating hormone. Leblond and Sue<sup>2</sup> observed that hypophysectomized rats concentrate much less of the administered tracer iodine in their thyroids than do intact controls. They also observed<sup>3</sup> that the thyroids of guinea pigs previously treated with thyroid-stimulating hormone trap more iodine in their thyroids than do untreated controls. On the basis of these observations, one might conclude that a primary action of the thyroid-stimulating hormone is to promote the collection and utilization of iodine. However, it might also be suggested that the increased collection of iodine by thyroids of animals previously treated with thyrotrophic hormone is independent of any direct action of the pituitary, but is related to an iodine want produced by a purging of the thyroid with TSH. This is suggested by the observations of Keating *et al.*,<sup>4</sup> who compared the anatomical changes with the loss and collection of radioactive iodine in cockerels treated with thyroid-stimulating hormone. In one study, they treated animals with one unit† of thyrotrophic hormone daily for 5 days. Groups of animals were sacrificed at the end of each 24-hour period, 4 hours after administering a tracer dose of radioactive iodine. The collection of radioactive iodine, thyroid weights and mean acinar cell heights were determined in each group of chicks. Results were calculated on the basis of per cent change over the controls. Though there were increases in the thyroid weights in the animals sacrificed at 24- and 48-hour intervals, a marked increase in weight

\* Present address: Memorial Hospital, New York, N. Y.

† Junkmann-Schoeller units.



was not manifest until after the third injection. The increase in mean cell height approached 200% in the first twenty-four-hour period, and 250% over the controls 48 hours after beginning treatment. There was no increase in the collection of radioactive iodine, however, until 48 hours after beginning treatment, at which time there was a hundred per cent increase in the collection of iodine. At the end of 96 hours there was a 500% increased collection of labeled iodine (see FIGURE 1).

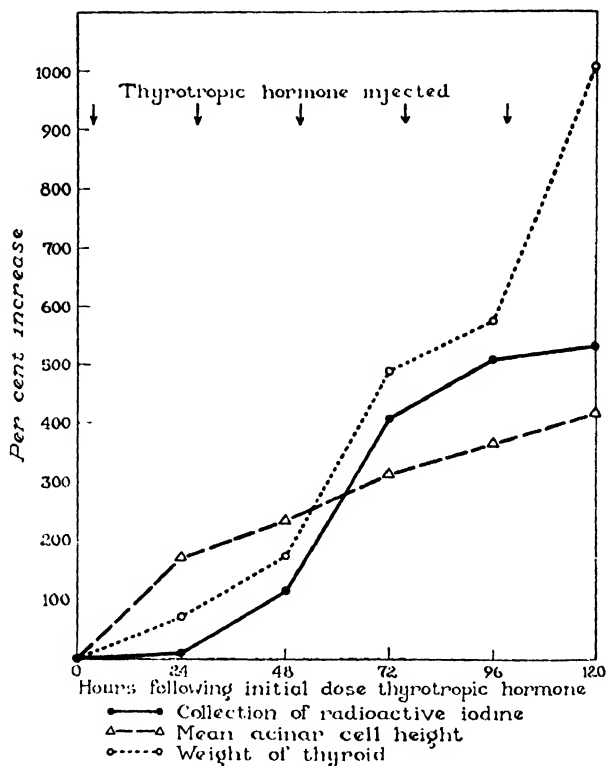


FIGURE 1. The effect of repeated doses of thyroid-stimulating hormone. Each group of animals was sacrificed 24 hours after the last injection of TSH and 4 hours after administering radioactive iodine.

In another group of experiments, these investigators followed the rate of iodine loss from the thyroids of newly hatched chicks. The chicks received a tracer dose of radioactive iodine before any food or water had been ingested. They then received one unit of thyrotrophic hormone daily for 3 days. One group was sacrificed every 24 hours up to 72 hours. The per cent of the previously administered radioactive iodine contained in thyroids at the time of killing was determined in each group. By comparing the values observed in the treated animals with those observed in chicks which received no TSH, the per cent loss of radio-iodine was determined. There was observed a 77% loss of the radioactive iodine 24 hours after the first injection of TSH. There was a loss of 93% and 96%

of the labeled iodine after 48- and 72-hour intervals (see FIGURE 2). Thus, it appears that the first changes to occur in TSH-treated chicks are a

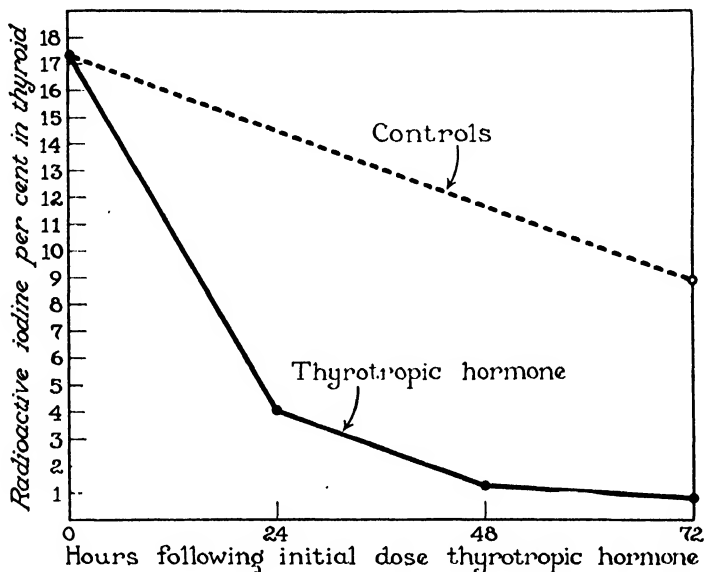


FIGURE 2. The effect of thyroid-stimulating hormone on the loss of iodine previously stored in the thyroid.

loss in thyroid iodine and an increase in the mean acinar cell height. The increased rate in collection of iodine did not occur until 24 hours later. In FIGURE 3, the per cent of iodine loss and the per cent of increased iodine collection are both plotted against time. From these curves, one might well obtain the impression that an increased iodine collection does not occur until after the thyroid has been purged of its iodine-containing hormone.

The observations of Morton, Perlman, Anderson and Chaikoff<sup>5</sup> would also indicate that the collection and utilization of iodine is not dependent upon an action of the thyrotrophic hormone. They observed that, though the thyroids of hypophysectomized rats collect less iodine than do those of the intact controls, they do collect a great deal more iodine than would be expected from simple diffusion. They observed that a large per cent of radio-iodine trapped by the thyroids of hypophysectomized rats is in the diiodotyrosine fraction. They found very little in the thyroxine fraction.

In another study done in our laboratory,<sup>6</sup> we have observed the rate of iodine loss and of changes in the mean acinar cell height after injecting one unit of thyrotrophic hormone. Cockerels five days of age, after having been on a diet of chick starter mash and tap water for 3 days, were given, by subcutaneous injection, one unit of thyrotrophic hormone. Ten treated and ten untreated controls were sacrificed every 6 hours for 48 hours.

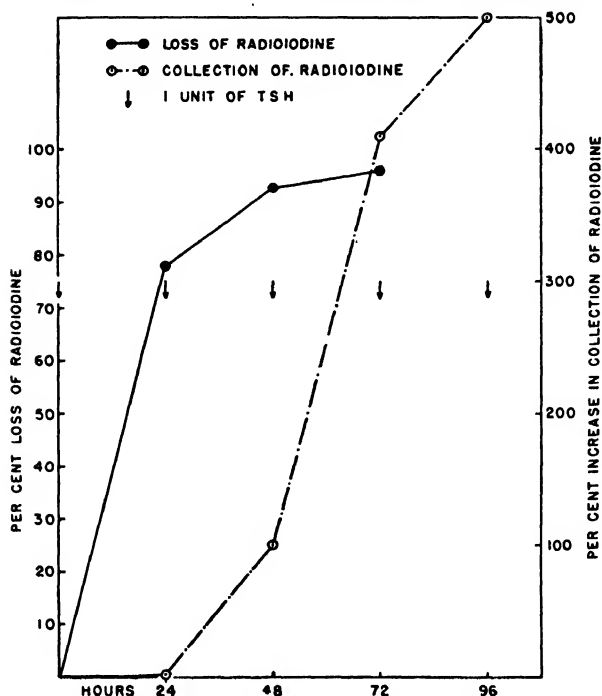


FIGURE 3. The per cent loss of radio-iodine and the per cent increase in collection of radio-iodine observed at varying time intervals after treatment with thyroid-stimulating hormone.

Four thyroid lobes were taken from each group of chicks and fixed in 10% neutral formalin. Paraffin sections 6 micra thick were prepared in the usual manner and stained with hematoxylin and eosin. Mean acinar cell heights were determined by the technique of Rawson and Slater.<sup>7</sup> The remaining 16 lobes of each group were equally divided and placed in vials containing 5 cc. of 2% KOH for digestion. Total thyroid iodines were done in duplicate on the digests by the method of Kendall<sup>8</sup> as modified by Astwood and Bissell.<sup>9</sup> A loss of thyroid iodine was observed in the treated groups as early as 6 hours after injecting the pituitary hormone. The maximum loss of iodine occurred 24 hours after injection of the TSH. After 30 hours, the direction of this curve was reversed. There was no demonstrable change in the mean cell height until 18 hours after injecting the TSH, 12 hours after the first significant change in thyroid iodine. The maximum change in mean cell height occurred at 30 hours, 6 hours after the peak in the iodine loss curve had been reached, following which there was a gradual return toward normal (FIGURES 4 and 5). Since it has been abundantly demonstrated that by far the major portion of iodine stored in the thyroid is in the thyroglobulin molecule, it is safe to say that the loss of iodine in these experimental animals is probably a loss of thyroid hormone. De Robertis<sup>10</sup> has reported that the colloid of thyroid follicles contains a proteolytic enzyme and that the pro-

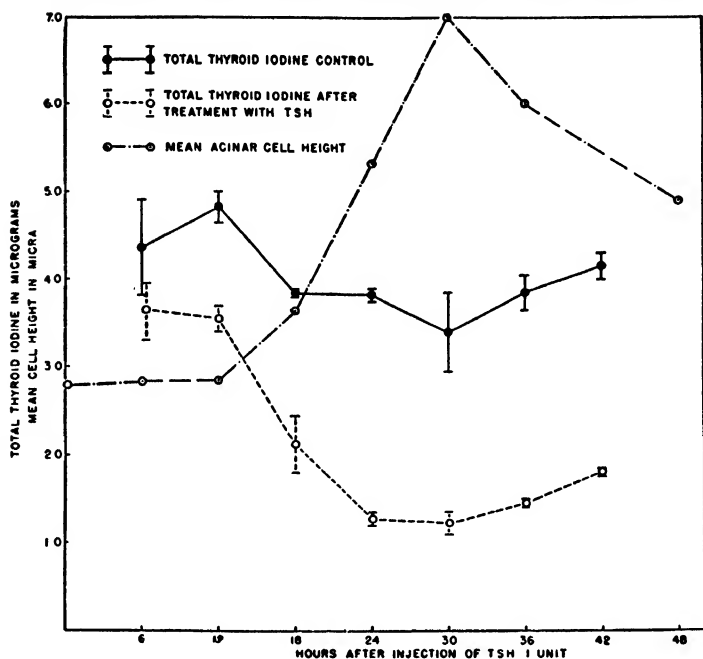


FIGURE 4. Thyroid iodine values and thyroid mean acinar cell-height measurements observed in cockerels at varying intervals following one injection of thyroid-stimulating hormone.

teolytic activity of droplets taken from thyroid follicles after the animals have been treated with TSH is increased. He has suggested the hypothesis that thyroglobulin is hydrolyzed in the lumen of the follicle by this proteolytic enzyme and that the smaller molecules resulting from this hydrolysis become available for transport through the follicular cells. If this concept is correct, it is quite likely that the loss of iodine observed by us in the thyrotrophic-hormone-treated cockerels is the result of an increase in proteolytic enzymes of the thyroid.

The effects that we have observed and the order in which they occur in the thyroids of animals treated with thyrotrophic hormone may be listed as follows: loss of thyroid iodine or thyroid hormone; increase in the thyroid acinar cell height; increase in thyroid weight; and finally an increase in capacity of the thyroid to concentrate iodine. It is an intriguing hypothesis that the primary effect of thyrotrophic hormone is to mobilize and release the thyroid hormone from the thyroid follicle and that the anatomical changes and increased avidity for iodine are secondary changes, not dependent on the pituitary. However, it is not proved.

Though the studies listed above would seem to indicate that at least one of the primary actions of thyrotrophic hormone is to liberate thyroid hormone from thyroid follicles, they do not throw any light on the mechanism of action of the thyroid-stimulating hormone. We have approached this by studying the effect of thyroid tissue on the thyroid-stimulating

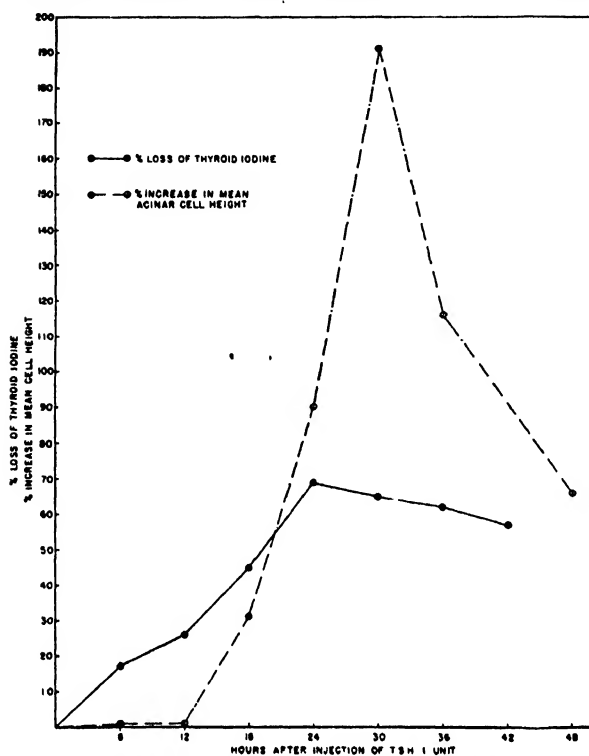


FIGURE 5. The per cent loss of thyroid iodine and the per cent increase in thyroid mean acinar cell height following one injection of thyroid-stimulating hormone.

effects of pituitary extracts. These studies were prompted by the clinical observations<sup>11</sup> that totally thyroidectomized patients excrete in their urines easily demonstrable amounts of active thyrotrophic hormone, whereas patients having untreated Graves' disease do not excrete active thyrotrophic hormone.

Tissue culture techniques were used in this study.<sup>12</sup> Roller bottles were coated with chicken plasma on which the tissue slices were clotted. The tissues were sliced and explanted in such bottles. The amount of thyroid tissue used in such experiments varied between 60 and 100 mgm. The tissues were then bathed in 15 cc. of Tyrode's solution containing thyroid-stimulating hormone in concentrations of  $\frac{1}{4}$ ,  $\frac{1}{2}$ , and 1 unit per cc. (total of 3.5 to 15 units per bottle). The bottles were rotated 15 revolutions per hour in an incubator at 38°C. for a three-day period. At the end of the three-day period the medium was withdrawn and assayed for thyroid-stimulating effect.

Assays for the thyrotrophic activity were carried out on female guinea pigs weighing 180 to 220 grams or on cockerels. They were quantitated, in most instances, by determining the mean acinar cell heights of thyroids removed from the test animals as described by Rawson and Starr<sup>11</sup> and

by Rawson and Salter.<sup>7</sup> In some instances when the assay was done on cockerels, the thyroid weights and thyroid iodines were also determined.

As one control, media containing the hormone in a concentration equivalent to  $\frac{1}{4}$  of a unit per cc. were incubated in plasma-coated bottles containing no tissue for three-day periods. Other control studies included three-day incubations of other tissue explants with media containing the hormone in a concentration of  $\frac{1}{4}$  unit per cc. The control tissues included rabbit adrenals, kidneys, livers, ovaries, pancreas, stomach mucosa, testes, thymus, and lymph nodes, and rat thymus and rat lymph nodes:

With these studies it has been consistently observed that there occurs a loss of thyroid-stimulating activity from the media exposed to explants of thyroid tissue (see FIGURE 6). With two exceptions, none of the control

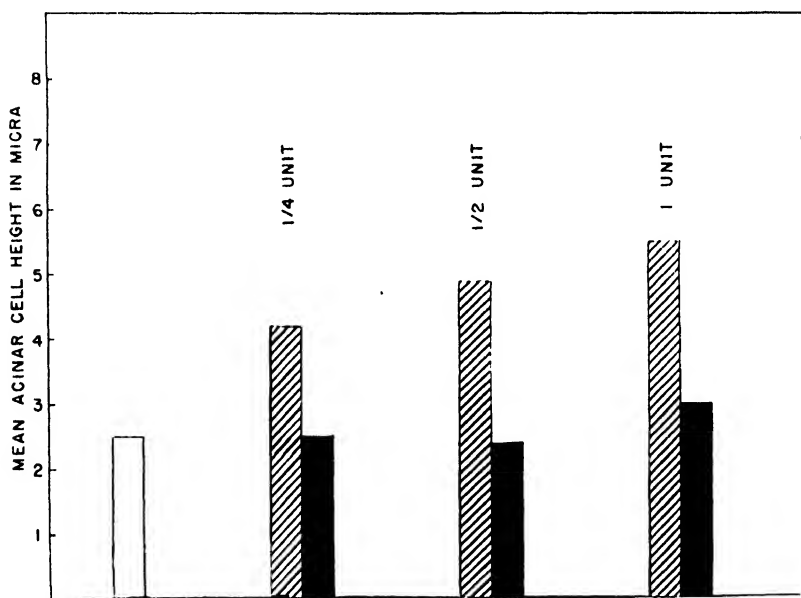


FIGURE 6. The mean acinar cell height of guinea pig thyroids after treatment with active and exposed (to explants of thyroid tissue) thyroid-stimulating hormone. The white column represents the thyroid mean acinar cell heights of untreated guinea pigs. The crossed-bar columns represent the thyroid mean acinar cell heights of animals treated with unexposed thyroid-stimulating hormone in the daily doses indicated. The solid black columns represent the thyroid mean acinar cell heights of guinea pigs treated with hormone following its exposure to explants of thyroid tissue.

tissues have been observed to have any inactivating effect on the thyroid-stimulating hormone contained in the bathing media (see FIGURE 7). The exceptions were thymic and lymph nodal tissue. Both of these tissues have been observed to inactivate the hormone but to a lesser degree than does thyroid tissue. Under optimal conditions, we have observed that a normal rabbit thyroid will inactivate as much as 12 units of TSH. Lymph and thymic tissue will inactivate as much as 5 units of the hormone (see

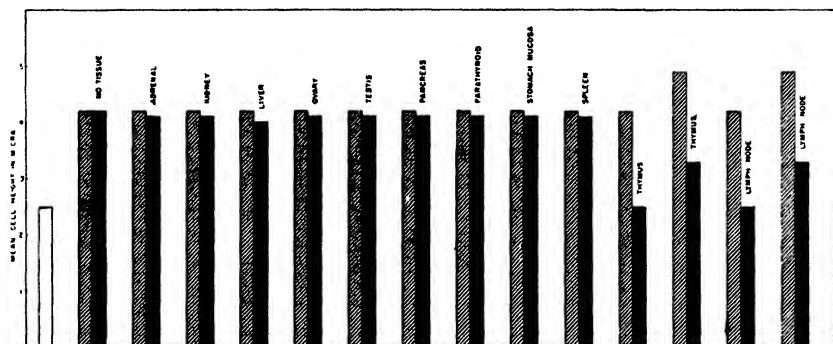


FIGURE 7. The mean acinar cell heights of guinea pig thyroids following treatment with  $\frac{1}{2}$  unit daily of unexposed thyroid-stimulating hormone illustrated by crossed-bar columns, and with the same amount of hormone following its exposure to explants of the tissues indicated, illustrated by the solid black columns. Thyroid-stimulating hormone in concentrations of both  $\frac{1}{2}$  and  $\frac{1}{4}$  units per cc. was exposed to explants of thymus and lymph nodes. The mean cell heights of untreated control guinea pig thyroids is indicated by the white column.

FIGURE 8). During the past 18 months, we have observed more variability in the capacity of rabbit thyroids to inactivate the hormone. On some occasions, one rabbit thyroid has been observed to inactivate as little as 3 to 4 units of the hormone. We suspect that this is probably due to the

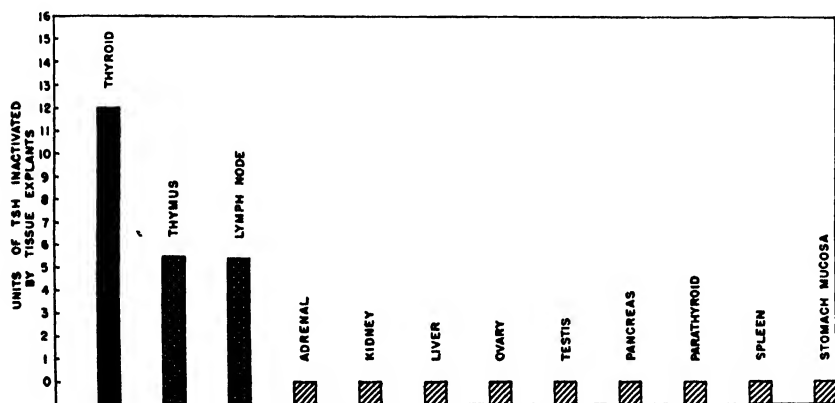


FIGURE 8. The amount of thyroid-stimulating hormone inactivated under optimal condition by the tissues indicated.

fact that we have been competing with the human consumers of rabbits for our animals. It has been suggested by some that this is due to changes in the nutrition of rabbits that we are able to purchase.

With Dr. Brown M. Dobyns,<sup>13</sup> we have observed that incubating pituitary extracts with thyroid tissue results in a loss of the exophthalmos-producing factor. Dobyns<sup>14</sup> has made daily measurements on the intercorneal distance of 300 to 400 gram thyroidectomized guinea pigs before and during treatment with pituitary extracts rich in thyrotrophic hormone. He has observed that thyroidectomy alone will cause a measur-

able increase in the intercorneal distance. However, the administration of certain extracts made from beef pituitaries (Antuitrin T, Parke Davis) produced an acute and marked increase in the intercorneal distances of thyroidectomized guinea pigs. Administration of purified extracts made from swine pituitaries resulted in much less exophthalmos. Using the same preparation that Dobyns found to be rich in the exophthalmos-producing factors we have explored the possibility of inactivating this factor of the pituitary by exposure to rabbit thyroid tissue.

In this study, we have pooled the media after exposure to thyroid tissue, concentrated it by precipitating the hormone with acetone, and then redissolved the hormone in water. Thyroidectomized guinea pigs were treated with an original equivalent of 25 units of exposed hormone per day for 7 days. There were two control groups, (1) untreated thyroidectomized guinea pigs, and (2) thyroidectomized guinea pigs treated with unexposed active thyroid-stimulating hormone which had been diluted to the same volume in Tyrode's solution, precipitated with acetone, and redissolved in an aqueous solution. Daily measurements of the intercorneal distance of each guinea pig were made by Dobyns's technique. The results were then plotted graphically (see FIGURE 9). At the end of

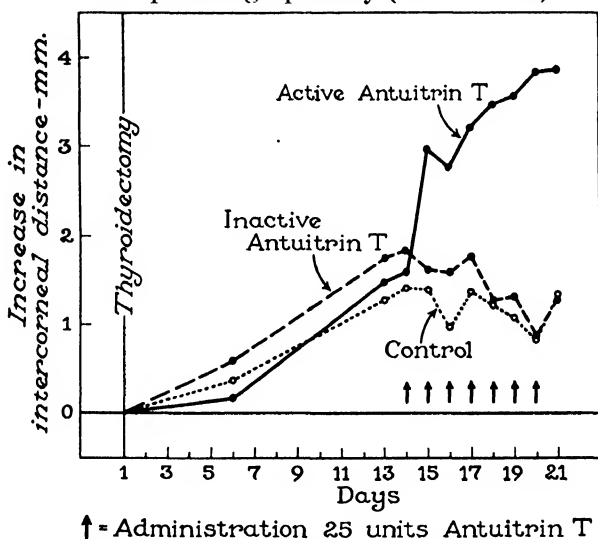


FIGURE 9. The effects of active and inactivated thyroid-stimulating hormone preparations on the intercorneal distances of thyroidectomized guinea pigs.

7 days, the animals which received unexposed thyrotrophic hormone had developed the classic picture of experimental exophthalmus. There occurred no exophthalmus in either the untreated guinea pigs or in the animals which received thyrotrophic hormone after it had been incubated with thyroid tissue. These observations may suggest that the exophthalmus-producing factor of the pituitary is closely related to TSH if not the same molecule.



In another study,<sup>15</sup> we have used tissue-culture techniques to determine the effect of normal and pathologic thyroid tissue removed from humans on the activity of thyroid-stimulating hormone. The same methods were used as those described above. In each experiment, approximately 150 to 200 mgm. of thyroid tissue were explanted. The thyroids of patients having Graves' disease were removed after obtaining a maximum response to treatment with iodine. The pretreatment basal metabolic rate levels of these patients ranged between +25 and +65. Non-toxic nodular goiters were also obtained from routine operations for such goiters. The basal metabolic rates of these patients were all below -10. Biopsy samples of normal human thyroid tissue were taken by Dr. Oliver Cope at operations for parathyroid tumors. It was observed that explants of nodular thyroids which, on histologic examination, showed colloid nodules, inactivated none of the exposed thyrotrophic hormone. Similar explants of normal human thyroid tissue were found to inactivate between 3 and 4 units of the hormone. The tissue explants taken from patients suffering with Graves' disease were found to inactivate between

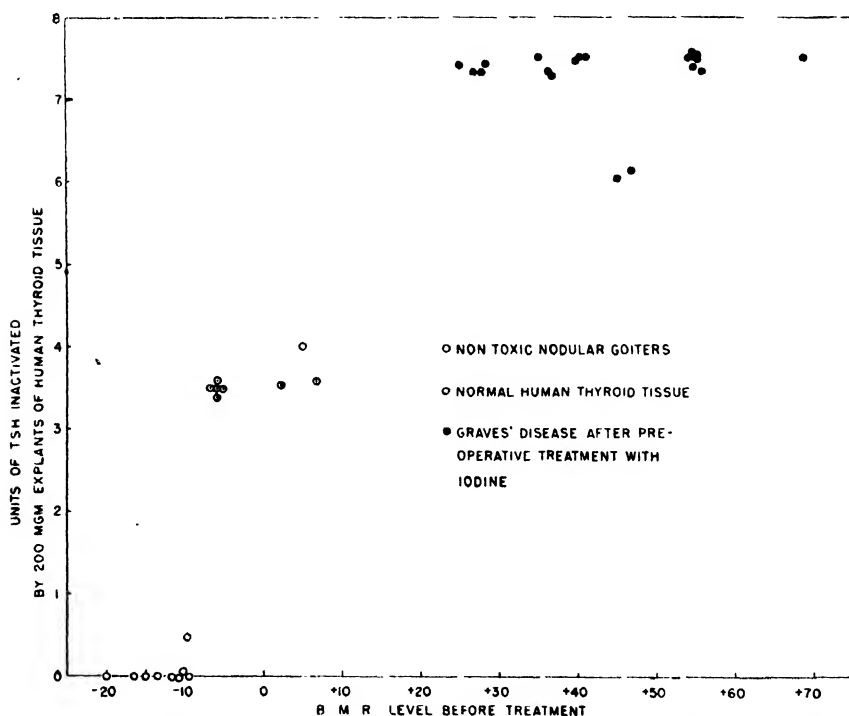


FIGURE 10. The quantity of thyroid-stimulating hormone inactivated by exposure to explants of normal and pathologic human thyroid tissue.

6 and 7.5 units of TSH (see FIGURE 10). It might be suggested that this increased reactivity between the thyroid of Graves' disease and the thy-

roid-stimulating hormone was due to an increased cell mass in these goiters. However, with few exceptions these glands were found to be involuted and two of them showed hyperinvolution. We have suggested that this reaction might explain our failure to demonstrate active thyroid-stimulating substances in the urine of untreated thyrotoxic patients. Indeed, we have suggested the hypothesis that this increased reactivity between the thyroid-stimulating hormone and thyroid tissue of patients with Graves' disease may be the key to an important etiologic factor in this malady.

The nature of this reaction between thyroid tissue and the thyrotrophic hormone is not known. It has been suggested that it is non-specific and possibly proteolytic in nature. Those who believe that the exophthalmos-producing factor of the pituitary is separate from the thyrotrophic factor may cite the observations that the exophthalmos-producing factor of pituitary extracts is lost following exposure to thyroid tissue in support of the thesis that the reactions we have observed are non-specific. However, by observing changes in the weight of testes taken from cockerels treated with pituitary extracts after exposure to thyroid tissue, we have demonstrated that inactivation of the thyroid-stimulating hormone by thyroid tissue is not paralleled by any loss of gonadotrophic activity. We can say then that this reaction is not a non-specific one affecting all pituitary hormones. It has been suggested that the inactivation of thyrotrophic hormone by thymic and lymph nodal tissue speaks against any specificity of this reaction. On the other hand, however, it has long been known that clinical Graves' disease is associated with a lymphoid hyperplasia and an enlargement of the thymus. Thus, that these tissues inactivate the thyrotrophic hormone as does the thyroid, may increase the significance of these observations that we have made.

We favor the hypothesis that the inactivation of thyrotrophic hormone by thyroid tissue is an oxidative phenomenon in which the thyroid-stimulating hormone contributes an integral part of its molecule to the metabolism of thyroid tissue. This theory is supported by the observation<sup>16</sup> that thyroid-stimulating hormone inactivated by exposure to thyroid tissue can be reactivated when treated with certain reducing agents which also are goitrogenic. In this study, explants of rabbit thyroid have been bathed in Tyrode's solution containing thyrotrophic hormone in a concentration of  $\frac{1}{4}$  unit per cc. for 3-day periods. The media were pooled and assayed. After the hormone had been demonstrated to be inactive, the pooled media were treated with reducing agents (in concentrations too small to produce goiters). We have observed that hormone demonstrated to be inactive, after incubation with thiouracil, recovers a major part of its original hormonal activity, *i.e.*, capacity to increase thyroid weight and to decrease thyroid iodine in the cockerel (see FIGURE 11). We have also observed that incubation of inactivated thyrotrophic hormone with 5 aminothiodiazole, 2 thiol, and 3 (phenylaminomethyl) thiazoline

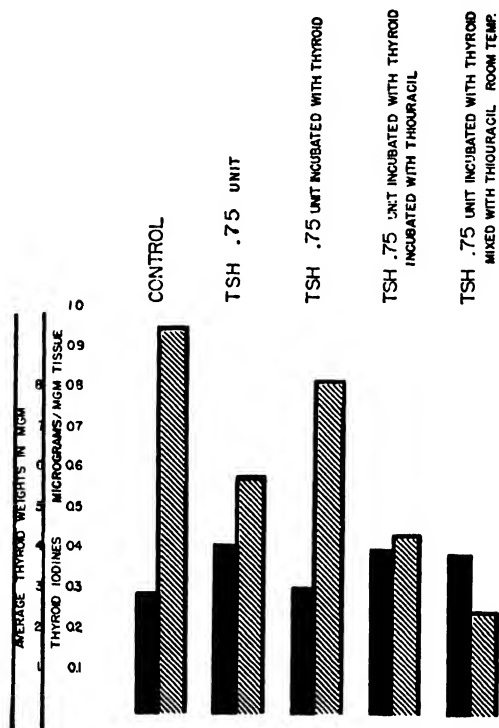


FIGURE 11. The effects of active thyroid-stimulating hormone following its exposure to rabbit thyroid tissue before and after treatment with thiouracil on the thyroid weights and iodine content in cockerels. The black columns illustrate thyroid weights, the crossed-bar columns thyroid iodines.

2 thione results in a recovery of the thyroid-stimulating action of the hormone mixtures. The latter two reducing agents in the intact rat have a goitrogenic activity comparable to that of thiouracil.

Further support for the thesis that the inactivation of the thyroid-stimulating hormone is an oxidative one is found in the observation reported by Dr. Albert<sup>17</sup> that this hormone loses its activity upon exposure to an oxidizing agent such as elemental iodine and that this inactivation of the hormone can be reversed when the inactivated hormone is exposed to a variety of reducing agents, most of which are goitrogenic.

These observations may indicate that the goitrogenic action of thiouracil and related agents is not wholly on the basis of thyroid inhibition. Indeed, we have observed that the administration of a standard amount of thyroid-stimulating hormone to chicks receiving thiouracil in the diet resulted in a marked augmentation of thyrotrophic potency when compared with controls receiving no goitrogen. Administration of thyrotrophic hormone after treatment with elemental iodine, which was inactive when given to controls, resulted in appreciable thyrotrophic effects in chicks receiving thiouracil (see TABLE 1).

In another group of experiments, we have studied the effect of iodide

TABLE 1

EFFECT OF ACTIVE AND INACTIVE TSH ON THE THYROIDS OF CHICKS  
ON NORMAL AND THIOURACIL DIET

Agents per chick*	Normal diet		Thiouracil diet	
	Thyroid weight mg.	Thyroid I μg. per mg.	Thyroid weight mg.	Thyroid I μg. per mg.
None	4.00	0.78	4.28	1.68
I-TSH†	3.70	0.76	5.80	0.08
TSII	6.50	0.07	9.14	0.00*

\* 100 Chicks total, 10 animals per subgroup. Average body weights varied from 45–50 gm. at end of experiment.

† I-TSH and TSH are abbreviations, respectively, for iodinated thyrotrophic extract, and active (untreated) thyrotrophic extract.

on the *in vitro* reaction between thyroid tissue and the thyrotrophic hormone. Explants of rabbit thyroid tissue were bathed in 15 cc. of Tyrode's solution containing  $7\frac{1}{2}$  units of thyrotrophic hormone ( $\frac{1}{2}$  unit per cc.), and iodine as sodium iodide in a concentration of 100 micrograms per cent. At the same time, similar explants of thyroid tissue were bathed in media containing the same amount of TSII without iodide. The degree of inactivation of the TSII was quantitated by microhistometric methods applied to thyroids of chicks after treatment with the various media. Complete inactivation of the hormone resulted from exposure to slices of thyroid tissue without iodide. However, when TSII was exposed in the presence of iodide, very little inactivation of the hormone occurred. It was calculated that, at most, less than one of the  $7\frac{1}{2}$  units was inactivated (see FIGURE 12). On the basis of these observations, we have

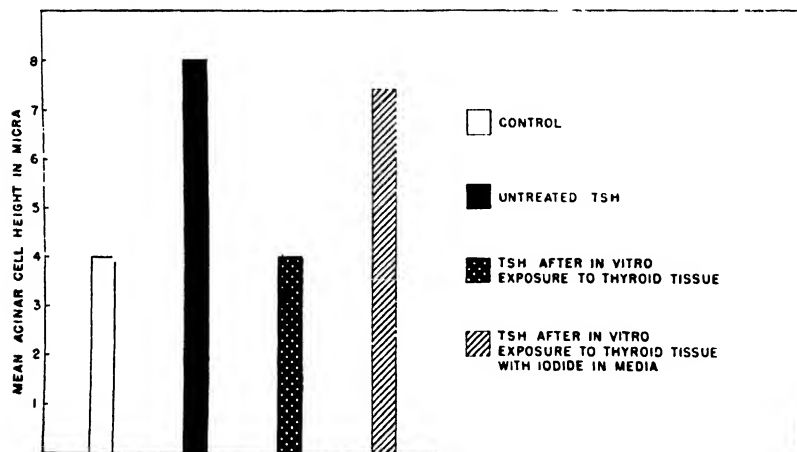


FIGURE 12. The effect of iodide in the bathing medium on the inactivation of thyroid-stimulating hormone by thyroid tissue.

suggested that the therapeutic effect of iodine, when administered to patients with Graves' disease, is due to a blocking effect of iodide to the reaction between the thyrotrophic hormone of the pituitary and the thyroid cell.

This theory is supported by the observation that administering iodine inhibits the histologic changes produced in hypophysectomized rats treated with thyroid-stimulating hormone. Male rats of the Sprague Dawley strain weighing 50 to 60 gm. were hypophysectomized. One month after operation they were treated with 8 units daily of TSH for 4 days. Iodine was administered to two groups of animals as sodium iodide in daily doses of 500 and 1000 micrograms during the period of treatment with thyrotrophic hormone. The animals were sacrificed on the 5th day. Thyroids of the rats proved to be hypophysectomized were fixed in (10% neutral) formalin. Paraffin sections were made and the mean acinar cell height of each thyroid was determined. Thyroid mean acinar cell heights of the untreated control rats averaged 3.8 micra; of rats treated with 8 units of TSH daily, 10.1 micra; of rats treated with TSH 8 units and NaI 1000 micrograms daily, 5.2 micra; of rats treated with TSH 8 units daily and NaI 500 micrograms daily, 5.4 micra (see FIGURE 13). Thus, it can be said that the reaction between thyrotrophic

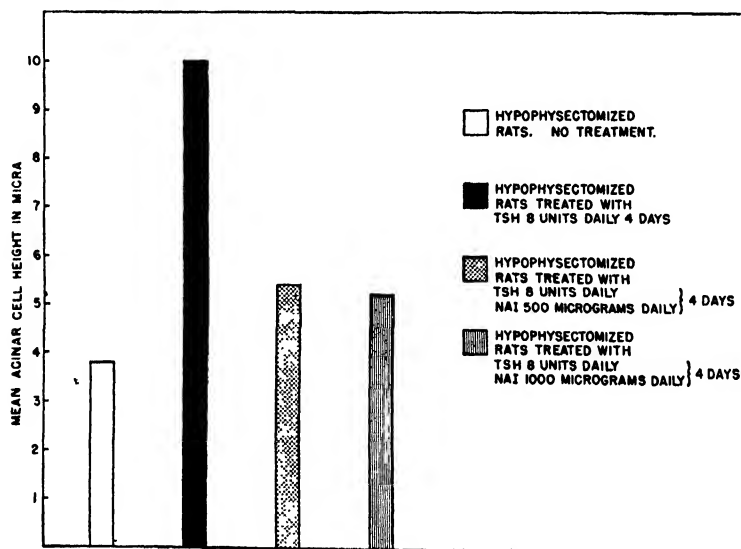


FIGURE 13. The effect of thyroid-stimulating hormone on the mean acinar cell heights of hypophysectomized rats when given with sodium iodide.

hormone and thyroid tissue can be prevented by iodides not only *in vitro* but also in the hypophysectomized rat. We have not explored the possibility of iodine inhibiting the pituitary's elaboration and secretion of the thyroid-stimulating hormone. However, we feel justified in advancing the hypothesis that at least part of the therapeutic action of iodine in Graves' disease is an inhibition to the reaction between thyroid tissue and the thyrotrophic hormone.

In a similar study<sup>20</sup> done on hypophysectomized rats, it has been demonstrated that the effect of thyroid-stimulating hormone on the

thyroid cell can also be inhibited by administering thyroxine. It has been demonstrated by several investigators that administering an excess of exogenous thyroid substance puts the recipient animal's own thyroid in a resting state.<sup>21</sup> It has also been demonstrated that the simultaneous administration of thyroid hormone with an active thyrotrophic hormone of the pituitary prevents, in part, the usual thyroid-stimulating effect of the pituitary extract.<sup>22</sup> It has been postulated that this effect of thyroid hormone was the result of suppressing the animal's own pituitary by the administered thyroid substance.<sup>23</sup> The present study was undertaken to determine whether the inhibitory action of thyroid hormone could be, in part, due to an effect on the thyroid cell. Male rats of the Sprague Dawley strain weighing between 70 and 90 grams were hypophysectomized, and the capacity of such animals to respond to thyroid-stimulating hormone was compared to that of similar animals receiving thyroxine. The thyroxine was administered in daily doses of 20 micrograms for 10 days. The thyrotrophic hormone was administered in daily doses of 4 Junkmann Schoeller units the last 4 days of each experiment. The responses of these animals to the thyroid-stimulating hormone were quantitated by determining the thyroid mean acinar cell heights and by measuring the thyroid's avidity for radioactive iodine. The mean acinar cell heights of untreated controls averaged 2.6 micra; of rats treated with TSH alone, 6.5 micra; of rats treated with thyroxine plus TSH, 4.8 micra; the per cent collection of the administered radioactive iodine by the unoperated untreated controls averaged 6.9%, the operated untreated controls, 0.4%, the hypophysectomized thyroxine-treated rats, 0.4%, rats treated with TSH alone, 7.8%, rats treated with thyroxine and TSH, 2.7%. Thus we have evidence that thyroxine interferes with the action of TSH on the thyroid cell. We have not studied the effect of thyroxine on the *in vitro* reaction between the thyroid cell and the thyroid-stimulating hormone.

### Summary

1. We have observed the following effects, in the order given, in thyroids of cockerels treated with thyroid-stimulating hormone: loss of thyroid iodine, increase in thyroid-acinar-cell increase in thyroid weight, and finally an increased capacity to concentrate radioactive iodine.

2. With *in vitro* techniques we have observed that the exposure of thyroid-stimulating hormone to explants of rabbit thyroid tissue results in a loss of the hormone's activity. Exposure of the hormone to explants of thymus and lymph nodes taken from rabbits and rats also resulted in loss of activity, but to a lesser degree than that caused by thyroid tissue. Incubation of the hormone with explants of adrenals, kidneys, livers, ovaries, pancreas, stomach mucosa, and testes from rabbits resulted in no loss of the hormone's thyroid-stimulating effect. Loss of the thyroid-stimulating effect upon exposure to thyroid tissue was not paralleled by

any loss of gonadotrophic activity of the pituitary extract used in this study.

Explants of normal thyroid tissue weighing 150 mgm. were observed to inactivate between three and four units of the thyrotrophic hormone. Similar explants taken from nontoxic colloid goiters failed to inactivate the hormone. Explants of the same weight taken from the involuted thyroids of patients with Graves' disease after treatment with iodine were observed to inactivate between 6 and 7.5 units of the hormone.

3. Hormone inactivated by exposure to thyroid tissue has been re-activated by certain reducing agents which are goitrogenic.

4. Iodide has been observed to inhibit the inactivation of the thyroid-stimulating hormone by slices of thyroid tissue. It has also been observed to inhibit the action of TSH on the thyroid of hypophysectomized rats.

5. The administration of thyroxine has also been observed to inhibit the action of the thyroid-stimulating hormone on the thyroid of hypophysectomized rats.

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# THE ADRENAL CORTEX\*

*Consulting Editor:* Robert Gaunt

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# NOTES ON THE HISTORY OF THE ADRENAL CORTICAL PROBLEM

By ROBERT GAUNT AND W. J. EVERSOLE

*Department of Zoology, Syracuse University, Syracuse, N. Y.*

Papers presented in symposia of this sort are prepared primarily by and for experts in the field, and are, therefore, concerned largely with new and specialized aspects of the subject. The authors of these notes, whose lot it was to arrange the program, have tried to present here a general historical introduction to the adrenal cortical problem. This was done with the hope that it might be helpful to those who have not had occasion to follow the rapidly changing currents of thought concerning adrenal function. The word "Notes" is used in the title advisedly. No attempt is made to be comprehensive, and bibliographic citation is limited largely to reviews and typical papers. We have tried to include brief mention of some of the important problems not covered elsewhere in this conference.

The progress of fruitful adrenal research in earlier years was as slow as it has now become rapid. In 1855, Thomas Addison<sup>2</sup> described the syndrome which bears his name, and attributed a life-maintaining function to the adrenal cortex. Brown-Sequard<sup>10</sup> the next year performed the first experimental adrenalectomies and verified the idea that the adrenals were essential for life. He even found, and thereby unfortunately reduced the credence that can be given this pioneer work, that the removal of one adrenal was sometimes fatal. His findings were quickly challenged by several workers,<sup>58, 26</sup> using mainly rats, who said that adrenal ablation did not necessarily cause death. During the remainder of the 19th century only sporadic work was done, but by about the end of the century, due to work such as that of Abelous and Langlois<sup>1</sup> and Boinet,<sup>7</sup> it was generally agreed that the adrenals were essential for life, and that apparent exceptions were due to varying amounts of accessory adrenal tissue in different species. The history of the subject until 1913 is well recorded in Biedl's monograph.<sup>6</sup>

An additional two decades or more were spent on the question of whether it was the adrenal cortex or medulla that was essential for life. This was largely because the discovery of epinephrine about 1900 diverted attention from any other possible adrenal secretion. Work such as that of Wheeler and Vincent<sup>84</sup> should have clinched the point, but acceptance of the idea was slow, and as late as 1927 Zwerner<sup>89</sup> initiated his studies on the adrenal with a paper showing that the cortex was the life-maintaining portion.

## *Adrenal Physiology of the 1920's*

In a *Physiological Reviews* paper of 1924, Stewart<sup>71</sup> wrote, "The cortex is the part of the adrenal essential to life. How it exercises its function is utterly unknown." He raised some question as to whether its essential role was due to an internal secretion. Six years later in the same journal, Britton<sup>8</sup> summarized an enormous amount of information from 311 publications, but was forced to the conclusion, "That the very meagre knowledge of corti-

coadrenal function does not lend itself at present time even to rational theorizing." Adrenal physiology got off to an unfortunate start, or rather, largely failed to start at all until the later '20s, because the inexpert animal surgery and care usually resulted (except in rats) in such quick death after adrenalectomy that only animals in a state of acute collapse were available for experimental study.

The perpetrators of poor surgery were eloquently chastised by Stewart<sup>71</sup> who, among his other contributions, left the brightest prose in the adrenal record. The insistence by Stewart and Rogoff on a high standard of surgical technique and animal care in adrenal work, and their demonstration that when such rules were practiced adrenalectomized dogs lived for periods of approximately 10 days,<sup>64</sup> rather than the shorter periods generally found before, was a contribution of great worth in inaugurating sound physiological studies.

As one looks back with the benefit of hindsight at the information on the adrenal cortex accumulated prior to 1930, the abjectly negative conclusions of the above-mentioned reviewers seem overdrawn. The lack, of course, was the key facts to which their available information could be related—among which was the lack of definite proof of the existence of an adrenal cortical hormone.

Placed in context at the present time, pre-1930 statements such as the following have a modern ring. It was, for instance, reported that there was a rise in the R.B.C. count in adrenal insufficiency,<sup>23</sup> an increase in viscosity and concentration of the blood,<sup>38, 49</sup> with a rise in urea<sup>16, 59, 61</sup> and in protein and total N,<sup>74</sup> possibly due to an increased susceptibility to histamine.<sup>13</sup> Some workers specifically associated these changes with an altered kidney function,<sup>16, 61</sup> and the existence of a low blood volume was recognized.<sup>38, 88</sup>

It was found that blood chlorides decreased after adrenalectomy,<sup>49, 63</sup> as did Na, pH, CO<sub>2</sub> tension and serum bicarbonate,<sup>75</sup> while total solids, K and Mg rose.<sup>5</sup> The administration of Ringer's solution was found to be beneficial to adrenalectomized animals<sup>72</sup> and the salutary effects attributed largely to Na salts.<sup>60</sup>

The possible relation of the adrenal cortex to cholesterol metabolism was extensively considered without conclusive results.<sup>61</sup>

Many workers found a low blood sugar at some stage of adrenal insufficiency, and it was known that liver glycogen levels were affected by adrenalectomy,<sup>61, 8, 80</sup> although there were slight if any changes in muscle glycogen.<sup>12</sup> The mechanisms involved were not known, and the distinction between cortical and medullary effects were sometimes vague.

Adrenalectomized animals were known to be susceptible to a host of stresses, drugs, toxins, etc.,<sup>80, 82</sup> but the tendency was more to try to interpret each susceptibility individually, rather than as expressions of a single mechanism as is now done.<sup>82, 69</sup>

The relative enlargement of the lymphatic tissues<sup>4</sup> and the increase in lymphocytes<sup>90</sup> after adrenalectomy were known. The importance of such observations as they relate to the resistance mechanisms of the body have, however, only been recently clarified.<sup>85</sup>

The relation of the adrenal to the adrenogenital syndrome excited great interest,<sup>22, 35</sup> and when cortical extracts were available attempts were immediately made to identify the "virilizing hormone." There was little initial success, but later the elaboration of androgenic steroids by the adrenal was established.<sup>87</sup> Stewart and Rogoff<sup>86</sup> had first shown that some influence, later identified as progesterone,<sup>21</sup> was present during pregnancy and after estrus in the dog, which prevented the development of adrenal insufficiency.

None of this work, however, was such as to permit a definite and convincing statement as to the function of the adrenal cortex. As the work accumulated, the symptoms of adrenal insufficiency became legion; obviously, few functions or organs were unaffected by adrenalectomy. In not a few cases, convictions and the language used to express them, were strong, even if fact and theory were shaky. Like Henry Ward Beecher's ministerial friend, the early adrenal workers were not without recourse to the use of shouts to overcome doubts. The bellicosity carried over into the '30s and enlivened physiological circles of that era. It was reminiscent of the verbal mayhem that troubled the pages of the American Journal of Physiology as the story of the function of the adrenal medulla evolved a few years earlier.

#### *Adrenal Physiology after 1930*

In 1930, the modern history of the adrenal cortex was opened. In that year, the preparation of the first good cortical extracts was announced. Final proof of the endocrine nature of adrenal cortex function was thereby afforded, and a completely new approach to physiological and clinical problems made available.

The status of the subject by 1937 is well summarized in the papers of the Cold Spring Harbor Symposium of that year.<sup>81</sup> Since then, a variety of reviews have treated general or specific aspects of the subject,<sup>82, 73, 39, 57, 82, 36, 40, 55</sup> that of Swingle and Remington<sup>80</sup> being the most comprehensive.

*Extraction of Hormones of the Adrenal Cortex.* Many investigators, over a long period of time, attempted to extract the cortical hormones. The problem was difficult for various reasons, among which was the low concentration of hormone in the gland and the difficulty of eliminating epinephrine as a contaminant. How credit should be divided for the success of these early efforts is, perhaps, a matter of opinion. Weakly active preparations may have been obtained before 1930.<sup>8</sup> It is generally agreed that Swingle and Pfiffner,<sup>77, 78</sup> and Hartman, Brownell, *et al.*<sup>33, 31</sup> first described methods based on lipid extraction procedures which proved to produce extracts that would keep adrenalectomized animals and Addisonian patients alive indefinitely. The Swingle-Pfiffner preparations were the ones most widely used. Hartman termed the active principle of the extracts "cortin."

Swingle and Pfiffner, in their first attempts to establish the potency of their preparations, adopted the rigorous procedure of demanding that they maintain adrenalectomized cats for 100 days. The extracts were weak by modern standards, and the use of about 5 cc. per day was necessary. They were also expensive, costing about a dollar per cc. to prepare. These 100-day cats, in which roughly five hundred dollars had been injected, were viewed with

some economic misgiving by those of us who, as graduate students in the Princeton laboratories at the time, were not sharing in the alleged prosperity of early 1929.

The first clinical trial with this extract in which a moribund patient was quickly revived was made in the Mayo Clinic by Drs. Rowntree and Greene.<sup>66</sup>

*Isolation of Active Principles of Extract of the Adrenal Cortex.* The physiological work of the early '30s was done with whole adrenal cortical extract, and under the assumption by most workers that it contained only one hormone. In 1936, however, work from the laboratories of Wintersteiner and Pfiffner, Reichstein, and Kendall began to show that a large number of steroids could be crystallized from cortical extract.<sup>57, 62, 40</sup> There was confusion at first, due to the lack of knowledge as to what to look for, concerning the nature or presence of biological activity in some of the compounds. 17-Hydroxy-11-dehydrocorticosterone, one of the potent steroids in affecting organic metabolism, was thought by Wintersteiner and Pfiffner to be inactive when they first isolated it, because of its weakness in life-maintaining activity, for which they were testing. Its biological properties were first revealed by Mason, Meyers, and Kendall,<sup>52</sup> using Ingle's muscle work test. Of the 28 adrenal steroids thus far isolated, only 6 are known to have corticoid activity, although some others have sex hormone activity. The latest work implies that the known active steroids exert qualitatively different effects (*cf.* Kendall's paper in this monograph). Most groups have found an amorphous fraction remaining in their extracts after known steroids have been removed which is highly active in maintaining life. Further study of the nature of this fraction is awaited with great interest.

The only adrenal steroid for which a cheap method of synthetic production has been available is desoxycorticosterone, generally used as its acetate (DCA). The small amounts of steroids in adrenal tissue preclude quantity production by extraction. Great difficulties were encountered in finding a synthetic method for the production of those steroids oxygenated at carbon-11. The recent discovery of such methods, effective if not cheap, as presented here by Dr. Kendall, is one of the most important events in adrenal history.

*Desoxycorticosterone—Is It a Cortical Hormone?* The biological properties of DCA have been extensively studied, and considerable physiological interpretation based upon them. Several of its properties are unique to known steroids. Reichstein is the only adrenal chemist to have obtained it from adrenal tissue, and he found it in very small quantity. There is basis for serious question as to whether this compound is secreted in significant quantities into the blood stream, and this creates the need for caution in interpreting physiological experiments based on its use.

*Early Experiments with Cortical Extract.* Armed with potent cortical extracts, investigators could make a fresh approach after 1930 to an analysis of adrenal function. One of the motivating hopes of the Princeton group, and probably others, in preparing such extracts was that overdosage phe-

nomena could be produced which would reveal the essential nature of cortical function more clearly than studies of adrenal insufficiency had done. The first attempts to produce such overdosage phenomena were made in the late Dr. Harrop's laboratory at Baltimore. As much as one hundred ml. of extract were injected into dogs, and 15 blood constituents were measured.<sup>28</sup> No effects at all were found! This disheartening result was the more unfortunate because slight variations in their technical procedure should have revealed clues of great importance. Many overdosage effects have since been seen.

*Electrolyte and Water Metabolism.* By 1932, however, the work leading to an appreciation of the role of cortical hormones in electrolyte and water metabolism was under way. Attention was recalled to the similarity of adrenal insufficiency and secondary shock, and the probable importance of the declining blood volume emphasized.<sup>79</sup> Loeb, *et al.*,<sup>43</sup> followed shortly by the Harrop group,<sup>30, 29</sup> noted in adrenal insufficiency a marked loss of plasma Na, due to renal wastage, and thus, apparently, accounted for the extracellular dehydration. More dramatically, Loeb<sup>42</sup> showed that NaCl alone would maintain an Addisonian patient.

With this report, most adrenal workers rushed to their laboratories with salt shakers in hand. It was generally found that salt would not maintain adrenalectomized animals indefinitely, but all agreed it was helpful. Rubin and Krick<sup>87</sup> contributed the highly useful technique of adding salt to the drinking water of adrenalectomized rats. Allers and Kendall<sup>18</sup> perfected a diet, high in a mixture of Na salts and low in K, which would maintain adrenalectomized dogs indefinitely. It was generally found that salt-treated animals could not withstand stress well. Harrison and Darrow<sup>27</sup> showed that a defect in renal tubular function accounted for the loss of Na and retention of K that occurred in adrenalectomized animals. For awhile, it was considered by some that *the* function of the cortical hormone was to act on the kidney and to regulate electrolyte and water excretion.

When it became apparent that the disordered Na metabolism could in itself hardly explain all of adrenal insufficiency, attention was focussed on K, particularly by Zwemer.<sup>91</sup> It had been shown<sup>5, 34</sup> that K might rise to toxic levels in adrenalectomized animals, and important details were added by others.<sup>80</sup>

Adrenal insufficiency was not destined to be so simply catalogued. The alterations in Na metabolism are undoubtedly important, as shown, if in no other way, by the therapeutic value of salt administration, but animals can recover from adrenal insufficiency without replacement of the Na if enough cortical hormone is made available.<sup>76</sup> The accumulation of K is likewise dangerous when it occurs, but many have found that a rise of plasma K is not necessarily prerequisite to adrenal insufficiency. The regulation of K metabolism is clearly a major adrenal function, but in the complex syndrome of adrenal insufficiency many other factors are involved as well.

When crystalline cortical steroids became available, it was soon shown that DCA was the most potent in causing a retention of Na and excretion of K, either in adrenalectomized or normal animals.<sup>80, 39, 36</sup> Most cortical (and



sex) steroids cause some Na-retention, with the exception of those oxygenated at C-17 which, transiently at least, may cause Na loss.<sup>83, 11, 37</sup> The sex steroids have a Na-retaining effect, but of these only progesterone is beneficial in prolonging the lives of adrenalectomized animals. The estrogens, for reasons not established, are toxic after adrenalectomy.<sup>21</sup>

It is doubtful now, as first thought, that the alterations in water metabolism which characterize adrenal insufficiency are entirely a passive response to the changing electrolyte levels. When DCA is given and Na retention induced, water, as might be expected, is retained as well. In the normal dog, and in hypophysectomized animals of other species, however, the chronic administration of DCA can lead to diabetes insipidus-like effects.<sup>60, 70, 86</sup> Cortical extract (which presumably contains, largely, the adrenal steroids oxygenated at C-11) is less effective, if active at all, in inducing this diuretic response.<sup>68</sup> On the other hand, a dramatic diuretic effect of cortical extract, or 17-hydroxy-11-dehydrocorticosterone, is seen if these substances are given to animals which have been hydrated by the forced feeding of water; this is to be correlated with the absence of normal water diuresis in adrenal insufficiency.<sup>19, 41</sup> Thus, the cortical hormones may be diuretic or anti-diuretic agents, depending upon various circumstances. A precise statement of these circumstances is not possible at present, but it is yet to be demonstrated that the alterations in water metabolism are entirely secondary to those of electrolytes.

*Carbohydrate Metabolism.* Early workers, as indicated before, noted changes in carbohydrate metabolism, such as low blood sugar and liver glycogen levels in adrenal insufficiency. Many workers considered these changes terminal, however, and a likely consequence of moribundity rather than an indication of a primary function of the adrenal cortex.

Dr. S. W. Britton, after 1930, began a long series of experiments leading him to a vigorous enunciation of the theory that the "prepotent function" of the adrenal cortex was to regulate carbohydrate metabolism.<sup>9</sup> His arguments were based primarily on two types of observations: first, that adrenalectomized animals developed acute hypoglycemia and low glycogen levels, and, second, that when normal fasted animals were given large doses of cortical extracts there was an increase in blood sugar and in both liver and muscle glycogen. Britton's story was viewed with considerable misgiving in the early '30s, partly because his interpretations were sweeping and seemed to relegate the concurrent work on electrolyte metabolism to a secondary role, and partly because of question raised as to some of the experimental details.<sup>46</sup> Most of this stormily debated work now fits into the picture of established fact when reinterpreted in light of modern knowledge of the multiple nature of cortical secretions, and modified to recognize the primary role of pituitary hormones in regulating muscle glycogen.

Long and Lukens provided the evidence resulting in widespread acceptance of the idea that in the regulation of organic metabolism the adrenal cortex had a role of direct rather than secondary importance. Their work started with the conclusive demonstration of the fact, first indicated by Hartman and Brownell,<sup>32</sup> that adrenalectomy ameliorated pancreatic diabetes,<sup>47, 44</sup>

and the finding by G. Evans, in Long's laboratory, that the adrenal cortices were involved in gluconeogenic processes.<sup>14</sup>

It was first thought that the action of cortical hormones was primarily to stimulate gluconeogenesis. Later the factor of an inhibition of carbohydrate utilization was demonstrated,<sup>46</sup> and has assumed increasing importance in interpretation of later work (*e.g.*, Ingle's paper in this monograph).

As quickly as crystalline adrenal steroids were made available, it became clear from reports of remarkable unanimity that the 11-oxygenated adrenal steroids alone exerted these dramatic effects on the metabolism of carbohydrates and related substances.<sup>80, 36</sup> While there is, in all probability, more common ground than has yet been understood between the relation of adrenal steroids to organic and electrolyte metabolism, it was clear by 1940 that these two aspects of adrenal function were probably under the control of different adrenal steroids. Attempts to explain them in terms of the action of one hormone had resulted in much of the earlier confusion. With these basic facts established, subsequent progress along these lines has been rapid, as indicated in the paper submitted here by Dr. Ingle.

#### *Other Aspects of Adrenal Function*

Because of the historical factors traced above, it has become customary to speak of adrenal function as having two main aspects: (a) a role in electrolyte metabolism, mediated primarily by a DCA-type of steroid, and (b) a role in carbohydrate and protein metabolism, mediated by the 11-oxygenated steroids. A third is sometimes and properly added, namely: a role, as yet ill-defined, of the androgenic adrenal steroids. Obviously, disorders in any of these categories involve a host of sequela, the primary or secondary nature of which is difficult to establish, and there are a great many well-established adrenal functions whose relation to such processes is not yet clear. Some examples follow.

*Stress.* The relation of the adrenal to stress is the keynote of this monograph; nearly all of the papers touch on some aspect of the subject. The older literature is replete with references to the fact that adrenalectomized animals are susceptible to stresses of almost every variety: cold, toxins, trauma, drugs, infections, etc. The earlier tendency was to try to relate a specific adrenal function to each of these susceptibilities.

It is clear now from the work reviewed in this monograph by Selye, Pincus, Sayers, and others that there is a non-specific physiological response to stresses in general, resulting in the acquisition of resistance, and that one important phase of that response is adrenal hyperfunction. The adrenal stimulation is the result of an increased output of pituitary adrenocorticotrophin, and without such pituitary-adrenal activation the resistance to all stresses is low.

The mechanism of the protective action afforded by cortical hormones is unknown for the most part, but one important aspect of it has been elucidated in recent years by White, Dougherty, and their associates. They have shown that increased cortical hormone levels induced by stress act on the lymphatic system to cause a dissolution of certain of its elements and a re-

lease into the blood of gamma globulins (antibody proteins) for use by the organism. Their work was presented at an earlier Academy conference<sup>86</sup> and hence was not repeated here. The steroids active in protecting against stress are primarily those oxygenated at C-11.<sup>80</sup>

The fact that cortical hormones are involved in the resistance to stress led logically to attempts, particularly during the war, to protect normal individuals against trauma, etc., by the administration of large doses of these hormones. In other words, an attempt was made to produce a super-normal resistance.

Results of such attempts were conflicting, but the weight of the greatest number of reports lies on the side of those who found the cortical hormones of slight, if any, usefulness in producing a hyper-resistance in animals or patients whose own adrenals were intact.<sup>80</sup> The basis for such findings, presumably, is, that in most cases, the adrenals can put out hormone at such an elevated rate in response to stress that still higher levels applied by injection is of little avail. A further theoretically possible factor is that, unless enormous doses of cortical hormone are administered, the effect of that given will merely be to inhibit release of pituitary adrenotrophin, which in turn would cut down the rate of adrenal secretion, and hence result in no net addition to the total amounts of hormone available to the organism.

There are, however, some conditions in which the administration of cortical hormones to intact animals will provide clearly a life-maintaining protection, e.g., in K intoxication,<sup>48</sup> in water intoxication,<sup>18</sup> and probably in insulin hypoglycemia.<sup>24</sup> It so happens that these are conditions of little clinical significance, or ones for which better therapies are available. They do indicate, however, together with much other evidence, that a search for the therapeutic usefulness of cortical hormones other than in frank Addison's disease is worth while.

*Circulation.* When adrenalectomized animals collapse under stress, a circulatory failure is the most conspicuous feature of the process. A role of the cortical hormones in maintaining the functional integrity of the circulatory system is clearly indicated. The mechanisms involved are not. Presumably, both the vascular musculature and capillary permeability are involved.<sup>80</sup>

*Reproduction and Lactation.* The ability of the adrenal cortices to secrete sex steroids is well established and the physiological and pathological implications have been extensively studied.<sup>55</sup>

A role of the cortical hormone in supporting lactation is also clearly seen,<sup>54</sup> and an association with the enzymology of milk production has been suggested.<sup>15</sup>

### *The Rat and the Adrenal Problem*

Adrenal experimental work is now done more on rats than on any other form. Until 1930, many investigators considered the rat unsuitable for experimental use, largely because, in the hands of most workers, and presumably because of the presence of adrenal accessories, this species survived adrenalectomy in 50 per cent or more of the cases.

In 1930, Pencharz *et al.*<sup>56</sup> reported that the rat was no exception to the rule that adrenalectomy was fatal. This statement attracted considerable attention, because the ease and economy of doing adrenal work with a rat colony was obvious. A flurry of papers was forthcoming, most of which agreed that rats rarely survive adrenalectomy.<sup>17</sup> This fact, together with the development of more and better micromethods for blood work and the conquest of surgical problems such as hypophysectomy and pancreatectomy, which frequently need to be combined with adrenalectomy, has made the rat suitable for most types of adrenal study.

The factors which account for the survival or non-survival of rats after adrenalectomy, however, are still far from completely defined. Some of the high survival figures reported by early workers may have been due to the use of scrap diets high in NaCl. It was shown<sup>20</sup> that distinct colony differences could account for variation in survival in some cases, and this has been seen now by several workers. The opinion is widely held, on the other hand, that indefinite survivals are due to incomplete adrenal removal or to failure to remove the peri-adrenal tissue, the presumed site of accessories. This argument is not sympathetically received by those of us who think we can do this simple operation, but who still see occasional instances of prolonged survival, particularly in adult adrenalectomized rats after some sustaining therapy has been withdrawn.

More perplexing, however, is the difficulty of modern investigators in actually finding the adrenal accessories (or fragments) which are supposed to account for survival. Two possibilities seem to exist. First, it may be that the rat, one of the most rugged and adaptable mammals, can, in rare cases, maintain itself indefinitely under mild laboratory conditions without cortical hormones or unusual dietary support. These long-surviving animals cannot withstand stress well. Secondly, McFarland<sup>53</sup> has recently stated that, if adrenalectomized rats are maintained on salt for awhile, functional cortical masses may then differentiate from coelomic mesothelium without forming all of the typical histological adrenal layers. This is reminiscent of the fact that the ovary in the adrenalectomized ground squirrel can assume cortical functions.<sup>25</sup> We believe we have seen in several instances the type of tissue to which McFarland refers, but, in the absence of detailed cytological study hesitated to call it adrenal tissue. The problem is worth further study.

### Conclusion

A list of the actions of cortical hormones could be continued almost indefinitely. It has been said that the ubiquitous finger of the pituitary reaches into almost all bodily processes (Evans). No less is this true of the adrenal cortex. The complete story of adrenal function could not be told in a two-day conference. It is the feeling and hope of those who arranged the program, however, that the papers presented here, while revealing a record of great accomplishment, will also presage a still more productive future.

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# THE PITUITARY-ADRENAL SYSTEM

By GEORGE SAYERS AND MARION A. SAYERS

*Department of Pharmacology, University of Utah School of Medicine, Salt Lake City, Utah*

The fluctuating needs of the tissue cells of the organism for cortical hormone, as it adjusts to environmental change or resists stress, are met by the combined activities of the pituitary and the adrenal cortex. It is convenient to consider the peripheral tissue cells, the pituitary, and the adrenal cortex as interdependent members of a system, the pituitary-adrenal system. Stress, applied to the peripheral tissue cells, results in activation of the anterior pituitary. The anterior pituitary in turn accelerates the secretory activity of the adrenal cortex through the elaboration of adrenocorticotrophic hormone (A.C.T.H.).

The first part of this paper will consider manifestations of adrenal cortical activity associated with a variety of environmental changes and stressful conditions. The second part will consider the mechanism whereby the great variety of non-specific stresses brings about an increase in the rate of elaboration of A.C.T.H. from the pituitary.

## *Indices and Classification of Adrenal Cortical Activity\**

With the availability of purified A.C.T.H.,<sup>35, 64</sup> it soon became evident that all of the morphological and chemical changes in the adrenal cortex which had been described as taking place following subjection of the animal to stress could be reproduced by the administration of this trophic substance.

A.C.T.H.,<sup>63</sup> as well as noxious stimuli,<sup>61, 69</sup> increases the gross size of the adrenal gland.

The sudanophilic substance, which most likely is cholesterol ester material, undergoes the same type of dynamic change following administration of A.C.T.H. as it does following subjection of the animal to acute stress.<sup>61</sup> These studies have made it possible to correlate the concentration of sudanophilic substance with the level of functional activity of the adrenal cortex and clarify much of the confusion which has existed in the literature on this subject.<sup>23</sup> During the period when the adrenal cortex is activated to increased activity by A.C.T.H. or stress, the sudanophilic substance of the gland is depleted. When the gland comes to rest after a period of activity this substance reaccumulates. These changes parallel the alterations in the cholesterol content of the gland (*vide infra*).

The high concentration and labile nature of cholesterol in the adrenal has been recognized for some time. The significance of this can now be more clearly understood. Physiological conditions which might be expected to create an increased demand for cortical hormones decrease the concentration of cholesterol in the adrenals.<sup>61</sup> Administration of a single dose of A.C.T.H. results in a prompt fall in the cholesterol content of the adrenal (FIGURE 1). Within three hours after administration of A.C.T.H., the cholesterol content

\* The experimental work presented in this first part was carried out in collaboration with Dr. C. N. H. Long, Dr. Abraham White, Edith G. Fry, Helen Lewis, and Dr. T. Y. Liang, of the Department of Physiological Chemistry, Yale University School of Medicine.

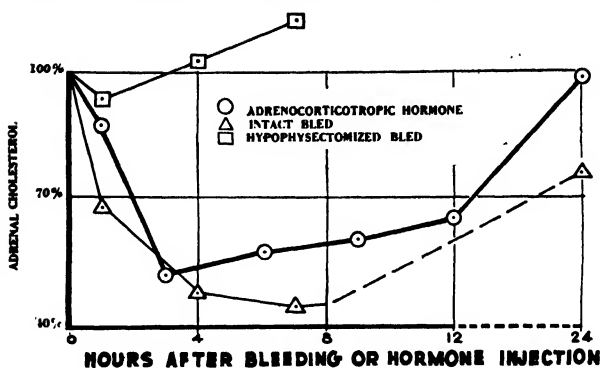


FIGURE 1. Changes in adrenal cholesterol following a single injection of A.C.T.H. (4 mg. per 100 gm. of body weight intraperitoneally) into intact rats and following an acute non-fatal hemorrhage of one hour's duration in intact and hypophysectomized rats. The slight changes which occurred in the cholesterol concentration of the adrenals of hypophysectomized rats are not significant.

of the gland has dropped to 50 per cent of its normal value. After twelve hours, the cholesterol begins to accumulate, and by twenty-four hours has returned to its original concentration. There is a striking similarity between these changes just described and those which follow subsection of the intact rat to a non-fatal hemorrhage. That it is the pituitary which mediates these dynamic changes in adrenal cholesterol is shown by the fact that they do not occur in the hypophysectomized animal exposed to this same stress.

It is ester cholesterol which is subject to the changes just described. Free cholesterol remains unchanged.<sup>39, 53, 65</sup> Other lipid constituents of the adrenal, such as neutral fat<sup>53</sup> and phospholipid,<sup>27, 39, 53</sup> remain unaltered at a time when the cholesterol content is markedly reduced.

The concentration of adrenal ascorbic acid, as well as that of cholesterol, is regulated by the pituitary. Administration of a single dose of A.C.T.H. results in a very prompt depletion of ascorbic acid from the adrenal of the rat (FIGURE 2). Within twelve hours, the original concentration is regained.

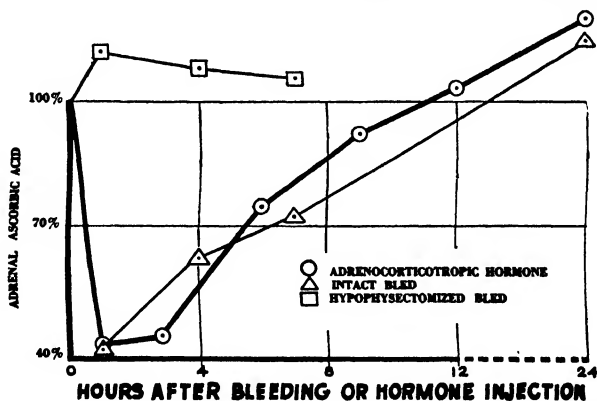


FIGURE 2. Changes in adrenal ascorbic acid following a single injection of A.C.T.H. (4 mg. per 100 gm. of body weight intraperitoneally) into intact rats and following an acute non-fatal hemorrhage of one hour's duration in intact and hypophysectomized rats. The slight increases in the ascorbic acid content of the adrenals of the hypophysectomized rats at 1, 4, and 7 hours are not significant.



Amounts slightly greater than normal are found after twenty-four hours.\* Following subsection of the intact rat to a severe but non-fatal hemorrhage, the absolute amount of the change in adrenal ascorbic acid as well as the time relationship of the change are strikingly similar to those alterations which follow administration of A.C.T.H. On the other hand, ablation of the pituitary leaves the ascorbic acid content of the adrenal inert and non-responsive when the animal is subjected to this same stress. However, injection of an exogenous source of A.C.T.H. will deplete the ascorbic acid of the adrenal of the hypophysectomized animal.†

Recently, both Levin<sup>34</sup> and Ludewig and Chanutin<sup>39</sup> have utilized changes in the cholesterol content of the adrenal to evaluate the response of this organ to several varieties of stress. It has been demonstrated that epinephrine,<sup>16, 36</sup> benzene,<sup>54</sup> estrogens,<sup>43</sup> ether,<sup>6, 33</sup> chloroform,<sup>33</sup> insulin,<sup>48</sup> diphtheria toxin,<sup>19, 71</sup> tetanus toxin,<sup>18</sup> anoxia,<sup>58</sup> infectious diseases,<sup>42</sup> atropine, nicotine, histamine, cold, heat, killed typhoid organisms, hemorrhage, dibenamine, and intraperitoneal administration of glucose<sup>65</sup> all deplete the ascorbic acid content of the adrenal. In the case of histamine, epinephrine, and cold, it has been possible to show that a relationship exists between the intensity of the noxious stimulant and the absolute drop in the concentration of ascorbic acid in the adrenal. The measurement of alterations in adrenal ascorbic acid has proved to be a particularly useful tool for the evaluation of adrenal cortical activity, since there is available a simple and accurate method for the determination of vitamin C.<sup>57</sup> ‡

An attempt has been made to bring some order out of the large number of varied and sometimes confusing reports on adrenal changes following exposure of animals to a multitude of noxious stimuli and environmental changes. The dynamic nature of the changes observed in the adrenal makes it essential that the following factors be taken into account in an interpretation of the data: (a) the intensity of the stimulation, (b) the duration of the stimulation, and (c) the time during or after stimulation at which the adrenals are analyzed. The responses of the adrenal cortex fall into six general types. The classification is intended as a guide to interpret functional activity of the adrenal cortex on the basis of changes in size, sudanophilic substance, cholesterol, and ascorbic acid of the gland. It is recognized that there are various grades of intermediate responses between these six types.

*Type 1. A sudden temporary period of stress* (FIGURE 3). In this situation, there is a sudden temporary increased demand for cortical hormones resulting in a temporary increase in pituitary adrenocorticotrophic activity. The sudanophilic substance, the cholesterol, and the ascorbic acid undergo rapid

\* The same dynamic changes in adrenal cholesterol and ascorbic acid have been shown to occur in the guinea pig.<sup>62</sup> As might be expected, however, reaccumulation of ascorbic acid is much slower in the guinea pig than in the rat. Rate of reaccumulation in the guinea pig is influenced by the exogenous supply of this vitamin.

† At the present time, vitamin C cannot be assigned a specific role in the processes concerned with the secretion or elaboration of adrenal cortical hormones. Depletion of ascorbic acid may be simply an expression of an overall acceleration of metabolic processes accompanying secretory activity in the gland.

‡ In small animals such as the rat, analysis of the whole adrenal is made. Separate analyses of adrenal medulla and adrenal cortex have demonstrated that the changes in cholesterol and ascorbic acid do occur in the cortex.<sup>63</sup> The authors have found the alteration in adrenal ascorbic acid to be the same measured by the method of Bessey,<sup>4</sup> which determines reduced ascorbic acid, as that measured by the method of Roe and Keuther,<sup>57</sup> which determines the total (reduced plus dehydro) ascorbic acid.

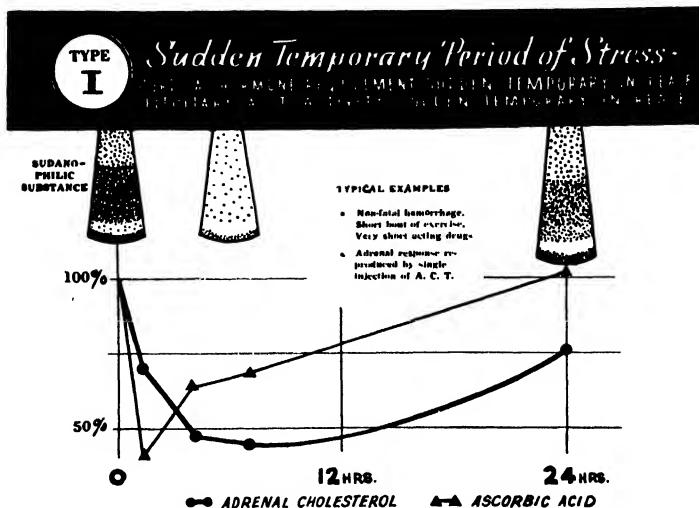


FIGURE 3. Type I adrenal response. The actual size of the adrenals is proportional to the total distance the diagrammatic section of the adrenal cortex extends below the upper bar. The changes in sudanophilic substance are taken from the observations of Engel, Winton, and Long.<sup>15</sup> The changes in the cholesterol, ascorbic acid, and size of the gland are those which have been observed to occur after a non-fatal hemorrhage of one hour's duration.<sup>42</sup>

depletion followed by a return of these substances to normal concentration as the animal recovers. A small increase in the size of the adrenal occurs. Typical examples of this type of adrenal response are produced by an acute non-fatal hemorrhage,<sup>62</sup> a short acting bout of muscular exercise,<sup>1, 14, 27</sup> and a single dose of very short acting drugs, such as epinephrine<sup>36</sup> or histamine. The changes in the adrenal characterized as Type 1 can be reproduced by the injection of a single dose of A.C.T.H.

It is evident that a dynamic process involving depletion and mobilization of cholesterol and ascorbic acid is taking place in the adrenals, and that the time of examination of this process is a variable which can quite adequately explain some of the apparent discrepancies which have been reported in the literature.

*Type 2. A very slow gradual change in the internal or external environment* (FIGURE 4). Here, there is a very slow gradual increase in the demand for cortical hormone which results in an equally gradual increase in pituitary adrenocorticotrophic activity. The concentration of sudanophilic substance, cholesterol, and ascorbic acid of the adrenal remain essentially unchanged. A gradual increase in size of the gland occurs, and it is apparent that new secretory units can be constructed at a rate sufficient to meet the increasing demands. Typical examples of this type are produced by fasting,<sup>40, 44, 47, 49, 50, 68, 72, 78</sup> seasonal changes in temperature, and pregnancy.<sup>2, 7, 55</sup>

*Type 3. An intense continuous stress ending in death* (FIGURE 5). There is a very great and continuous demand for cortical hormone, resulting in a maximum rate of elaboration of A.C.T.H. from the pituitary. The concen-

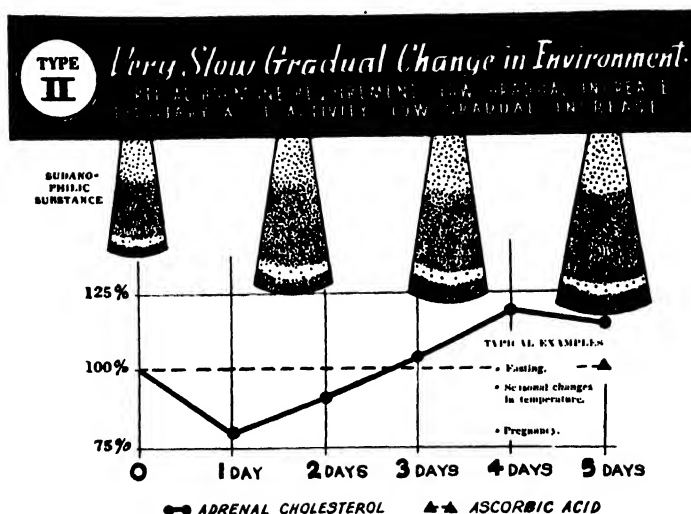


FIGURE 4. Type 2 adrenal response. The actual size of the adrenals is proportional to the total distance the diagrammatic section of the adrenal cortex extends below the upper bar. The data on sudanophilic substance are taken from the observations of Whitehead<sup>16</sup> on fasting animals. The data on size and cholesterol concentration of the gland represent the actual changes found by Ludewig and Chanutti<sup>17</sup> to occur in fasted rats. The changes which did occur in the cholesterol content of the adrenal are not considered significant. In the case of adrenal ascorbic acid, no data are available for periods of fasting less than five days. However, a fast of six days,<sup>18</sup> seven days,<sup>17</sup> or even 10 days<sup>18</sup> produces no significant change in adrenal ascorbic acid.

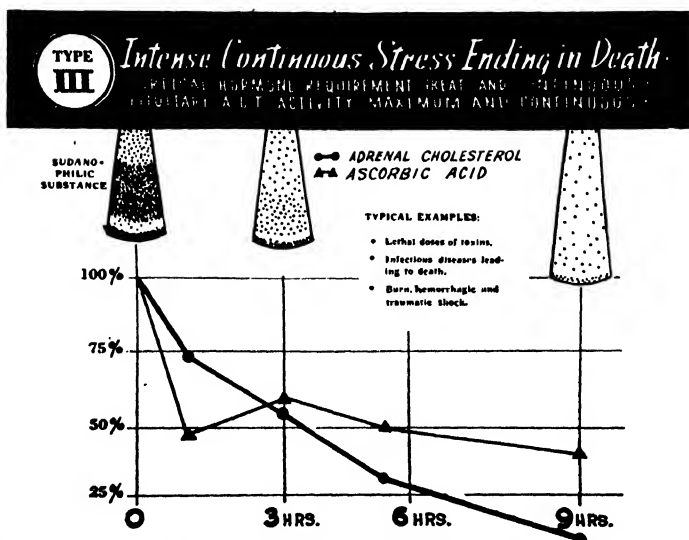


FIGURE 5. Type 3 adrenal response. The actual size of the adrenals is proportional to the total distance the diagrammatic section of the adrenal cortex extends below the upper bar. The data on sudanophilic substance are taken from the observations of Engel, Winton, and Long<sup>19</sup> on hemorrhagic shock in the rat. The changes in size, cholesterol, and ascorbic acid concentration of the gland are those which occur in rats subjected to hemorrhagic shock.<sup>20</sup>

trations of sudanophilic substance, cholesterol, and ascorbic acid fall rapidly and remain at a very low level until the time of death. The gland is laying down secretory units at a maximum rate, as evidenced by its marked hypertro-

phy. The amount of this hypertrophy is proportional to the time which elapses between the onset of the stress and death. Typical examples of this type of adrenal response are produced by lethal doses of toxins,<sup>8, 13, 45</sup> infectious diseases leading to a fatal outcome,<sup>3, 5, 11, 13, 17, 20, 28, 30, 31, 32, 38, 42, 60, 67, 75, 76, 79</sup> burn,<sup>9, 21, 52, 73</sup> and hemorrhagic<sup>13, 15, 62</sup> and traumatic<sup>12, 46</sup> shock.

*Type 4. Recovery from a period of severe stress or adaptation to stress* (FIGURE 6). In this case, the demand for cortical hormone is reduced follow-

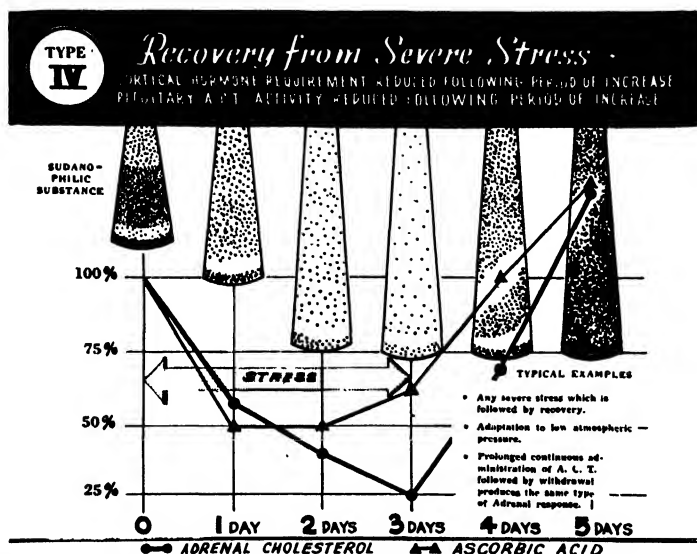


FIGURE 6. Type 4 adrenal response. The actual size of the adrenals is proportional to the total distance the diagrammatic section of the adrenal cortex extends below the upper bar. The changes in size and cholesterol concentration in the gland are taken from the data of Ludewig and Chanutin.<sup>39</sup> The changes in sudanophilic substance<sup>41</sup> and ascorbic acid concentration<sup>42</sup> are changes which take place when an animal is administered A.C.T.H. for a period of three days and then allowed to recover.

ing a period of stress, and the pituitary returns to a low state of functional activity. During the period of stress, the adrenal becomes enlarged and depleted of sudanophilic substance, cholesterol, and ascorbic acid. Upon removal of the stress, these metabolic constituents accumulate in a manufacturing plant which is over-expanded for the needs of the organism under optimal conditions of the environment. The changes in adrenal size and cholesterol concentration depicted in FIGURE 6 are taken from the data of Ludewig and Chanutin.<sup>39</sup> These investigators determined the concentration of cholesterol in the adrenal each day for several days following the administration of  $\beta$ -ethyl vesicants. The initial depletion of the cholesterol content of the gland represents the toxic phase of the response. The return to normal and subsequent increase of concentration above normal represent the recovery phase.

This type of adrenal response also occurs when an animal adapts to a continuously applied noxious stimulus. Initially, there is depletion of adrenal constituents, but, as specific adaptations are made, the requirement

for cortical hormone is reduced and the gland returns to a lower state of functional activity. For example, Darrow and Sarason<sup>10</sup> demonstrated lipid depletion of the adrenal cortex during the first five days of exposure of rats to anoxia, followed by restoration of lipid material on the seventh and eighth days of exposure.

These changes can be reproduced by the continuous administration of A. C. T. H. for a period of a few days, followed by cessation of administration of the hormone.

It is of interest to note that the phase of accumulation of cholesterol and ascorbic acid in a hypertrophied gland corresponds to the period of increased crossed resistance which follows stress.<sup>37</sup>

*Type 5. Dysfunction of the pituitary attended by hypersecretion of A.C.T.H.* In this case, the organism has no need for increased amounts of cortical hormone, but, due to some malfunction of the pituitary, the adrenal cortex is stimulated to increased secretory activity. The adrenal is maximally increased in size and continuously depleted of its sudanophilic substance, cholesterol, and ascorbic acid. The clinical example of this is Cushing's disease. The picture can be reproduced in the laboratory by chronic administration of large doses of A. C. T. H.

*Type 6. Dysfunction of the pituitary attended by hyposecretion of A. C. T. H.* In this case, the adrenal cortex atrophies as a result of pituitary hypofunction. The sudanophilic material, the cholesterol, and ascorbic acid of the adrenal become inert and non-responsive to changes in the internal and external environment. A clinical example of this type is found in panhypopituitarism. In the laboratory, it is reproduced by hypophysectomy.

*Summary.* Data have been accumulated on the effect of A.C.T.H. on the morphology and chemistry of the adrenal gland. These studies have made it possible to correlate levels of functional activity of the adrenal cortex with histological and chemical changes which have been described as occurring in this gland in a multitude of varied stresses and environmental conditions. A classification of the various adrenal responses into six types has been made.

#### *Regulation of Pituitary Adrenocorticotrophic Activity\**

Whereas the nature of the regulatory control of the pituitary over the adrenal cortex has been definitely established, little is known in regard to the humoral or nervous changes produced by stress which result in rapid elaboration of A.C.T.H. from the hypophysis. Contributions to our knowledge of this problem have been few. Ingle<sup>22</sup> was able to demonstrate an

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Grateful acknowledgement is made to Dr. Louis S. Goodman for his able advice and criticism during the course of this investigation.

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increase in adrenal size following a 12-hour period of muscular exercise in the rat. Hypertrophy did not occur in those animals treated with adrenal cortical extract during the period of exercise. The experiment may be interpreted to mean that administration of cortical extract abolished the normal stimulus to pituitary activity, namely: a decrease in concentration of cortical hormone(s) in the body fluids. According to Uotila,<sup>74</sup> the adrenal cortical hypertrophy which occurs in rats exposed to cold is not influenced by transection of the infundibulum. The following approach to this problem has been made possible through the availability of a new technique for measuring pituitary adrenocorticotrophic activity.

*Changes in the concentration of adrenal ascorbic acid reflect changes in pituitary adrenocorticotrophic activity.* It has been demonstrated that the concentration of ascorbic acid in the adrenal is under the specific regulatory control of the A.C.T.H. of the anterior pituitary. Part of the evidence upon which this conclusion is based is presented in FIGURE 7. Administra-

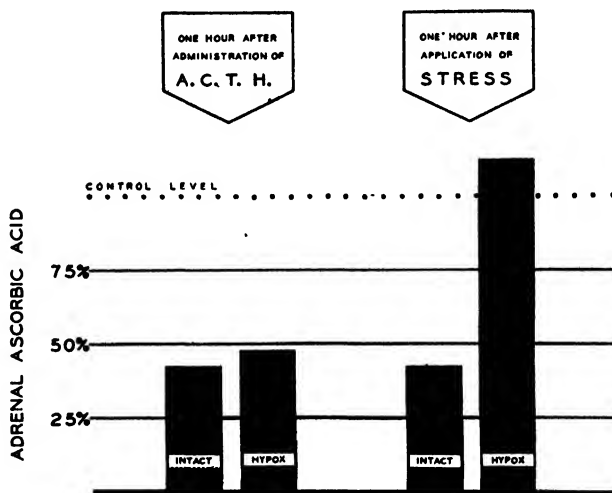


FIGURE 7. Effect of A.C.T.H. and stress (non-fatal hemorrhage of one hour's duration) upon the concentration of ascorbic acid in the adrenals of intact and hypophysectomized rats. The level of the dotted line represents the concentration of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dotted line is proportional to the amount of A.C.T.H. elaborated by the pituitary or to the amount of this trophic hormone injected intraperitoneally. Hypophysectomized is abbreviated to hypox.

tion of a purified preparation of hog A.C.T.H., chemically homogeneous and free of other known pituitary activities, depleted the ascorbic acid content of the adrenals of both the intact and hypophysectomized rat to less than 50 per cent of the control level within a period of one hour following injection. However, whereas application of stress produced the same marked and rapid depletion in the case of the intact rat, it had no influence upon the level of the vitamin in the adrenals of the hypophysectomized rat. The evidence leaves little doubt that the level of ascorbic acid in the adrenal is a reflection of the rate of elaboration of A.C.T.H. from the pituitary.

The alteration in the concentration of ascorbic acid of the adrenal offers

certain advantages as a measure of pituitary adrenocorticotrophic activity. Rapidly fluctuating rates of elaboration of A.C.T.H. can be detected. As can be seen from FIGURE 7, marked depletion of adrenal ascorbic acid took place within an hour following subsection of the intact rat to bleeding. Furthermore, the quantitative nature of the method has made it possible to demonstrate that *the rate of elaboration of A.C.T.H. from the pituitary is proportional to the intensity of stress to which the animal is subjected*. Reducing the temperature of exposure successively from  $7 \pm 1^\circ\text{C}$ . to  $4 \pm 1^\circ\text{C}$ ., to  $-2 \pm 1^\circ\text{C}$ ., and to  $-8 \pm 1^\circ\text{C}$ . brought about correspondingly greater reductions in adrenal ascorbic acid (FIGURE 8). Likewise, when the dose of

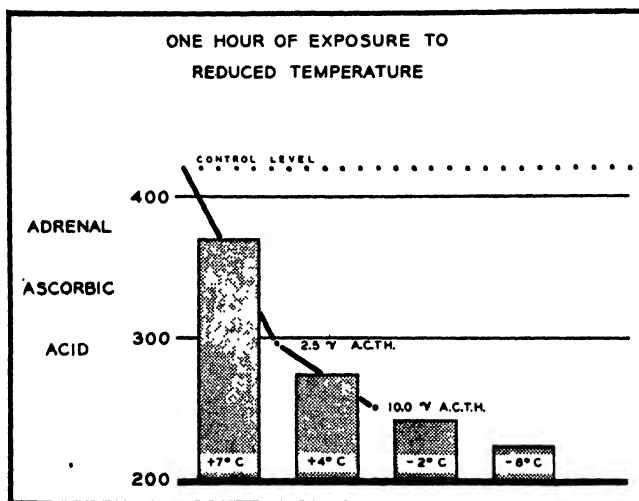


FIGURE 8. The effect of exposure of rats to varying temperatures upon the concentration of ascorbic acid in the adrenals. The level of the dotted line represents the concentration (mg. per 100 gm. of fresh tissue) of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dotted line is proportional to the amount of A.C.T.H. elaborated by the pituitary. Superimposed upon the bar chart is a graph showing the relation between dose of A.C.T.H. (hog) and adrenal ascorbic acid.

injected histamine was increased successively from 0.25 mg. to 0.5 mg., to 1.0 mg., and to 10.0 mg. per 100 gm. body weight, correspondingly greater reductions in the ascorbic acid content of the adrenals took place (FIGURE 9). Superimposed upon the bar charts (FIGURES 8 and 9) is a graph illustrating the direct relationship between the dose of hog A.C.T.H. administered and the reduction in adrenal ascorbic acid. The amount of hormone elaborated by the rat pituitary following exposure to stress can be estimated in terms of activity equivalents of hog A.C.T.H.

*Pretreatment with adrenal cortical hormone prevents the depletion of adrenal ascorbic acid which otherwise follows exposure to cold.* A rapid elaboration of A.C.T.H. from the pituitary follows the exposure of the rat to cold. As can be seen from FIGURE 10, exposure of the rat to  $3^\circ\text{C}$ . for one hour resulted in a marked depletion of adrenal ascorbic acid. Administration of cortical hormone one hour before the beginning of exposure prevented this depletion.

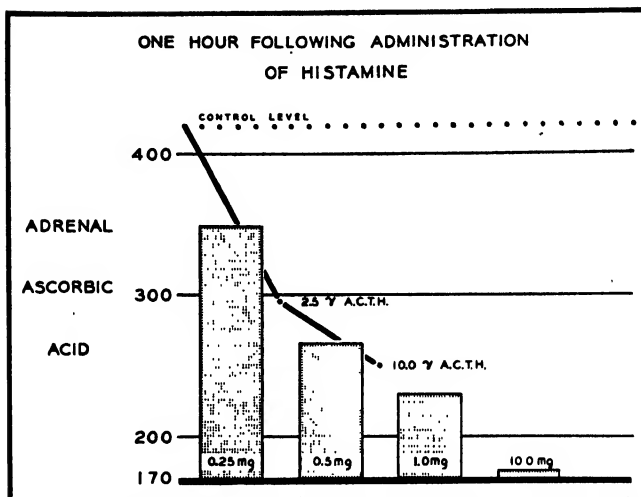


FIGURE 9. The effect of administration of various doses of histamine acid phosphate upon the concentration of ascorbic acid in the adrenals. The level of the dotted line represents the concentrations of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dotted line is proportional to the amount of A.C.T.H. elaborated by the pituitary. Superimposed upon the bar chart is a graph showing the relation between dose of A.C.T.H. (hog) and adrenal ascorbic acid.

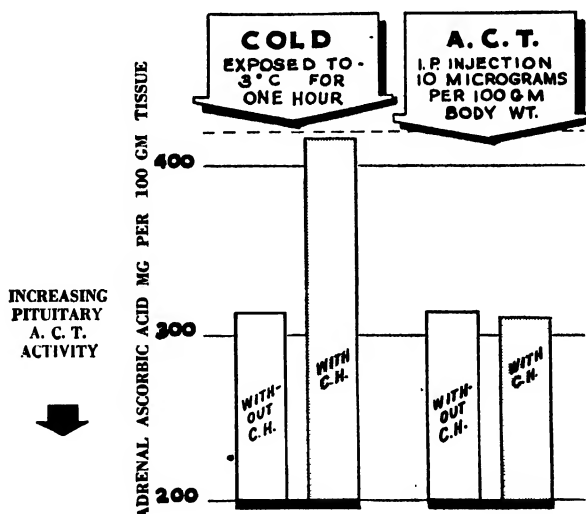


FIGURE 10. Mode of action of cortical hormone in preventing reduction of adrenal ascorbic acid upon exposure of the rat to stress. The level of the dashed line represents the concentration of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentration of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dashed line is proportional to the amount of A.C.T.H. elaborated by the pituitary or to the amount of this trophic hormone injected intrapituitarily.

A dose of A.C.T.H. was determined which produced the same drop in adrenal ascorbic acid as exposure to 3°C. for one hour. Whereas pretreatment with adrenal cortical hormone prevented the reduction in adrenal as-



corbic acid which otherwise follows exposure to cold, it had no effect whatsoever upon the adrenal stimulating effect of the exogenous source of A.C.T.H. (FIGURE 10). Hence, the locus of the inhibitory action of adrenal cortical hormone is not the adrenal cortex itself. By elimination, it would appear that adrenal cortical hormone acts to suppress elaboration of A.C.T.H. from the anterior pituitary.

*The phenomenon of inhibition of pituitary adrenocorticotrophic activity by cortical hormones is of a general nature.* Not only exposure to cold, but also application of heat, injection of histamine, epinephrine, and killed typhoid organisms brought about a reduction in the concentration of ascorbic acid in the adrenal of the rat (FIGURE 11). This reduction in the vitamin C

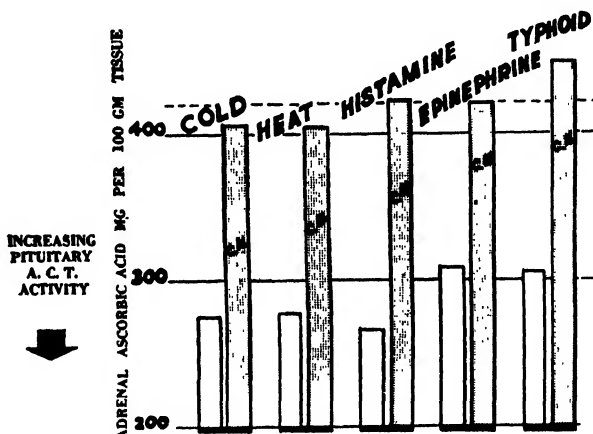


FIGURE 11. General nature of the phenomenon of pituitary inhibition by cortical hormones. The level of the dashed line represents the concentration of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dashed line is proportional to the amount of A.C.T.H. elaborated by the pituitary. The unshaded columns represent the changes which took place without the cortical hormone treatment and the shaded columns the changes with cortical hormone treatment. The concentrations of ascorbic acid in the adrenals of rats treated with cortical hormones as represented by the shaded columns are not significantly different, in any case, from the concentration of ascorbic acid in the adrenals of untreated rats.

content of the adrenals indicates that each one of these stresses, acting for a period of one hour, stimulated the anterior pituitary to elaborate A.C.T. H. On the other hand, if the animals were administered cortical hormone immediately prior to the application of the stress, no reduction in the concentration of adrenal ascorbic acid took place.

The administration of cortical hormone suppressed the enhanced pituitary adrenocorticotrophic activity which otherwise accompanies environmental change (heat, cold), drug administration (histamine, epinephrine), and bacterial intoxication (injection of a suspension of killed typhoid organisms). Hence, if the animal is furnished with an adequate exogenous source of adrenal cortical hormone during exposure to any one of a number of stresses, the normal stimulus to adrenal cortical activity is abolished. It would appear reasonable to suppose that the great variety of non-specific stresses increases pituitary adrenocorticotrophic activity by a common mechanism, namely, by increasing the requirement of the peripheral tissue

cells for cortical hormone(s). The anterior pituitary responds to the decrease in concentration of cortical hormone in the body fluids by increasing the rate of elaboration of A.C.T.H. It is recognized that in addition to the pituitary itself, some other intermediary structure may be involved. The hypothalamus, for example, rather than the pituitary, might interpret the blood levels of cortical hormone and make the appropriate adjustment in pituitary adrenocorticotrophic activity *via* nervous pathways. As mentioned previously, however, Uotila<sup>74</sup> found that section of the infundibulum did not interfere with the hypertrophy of the adrenal cortex which accompanies chronic exposure to cold.

*Certain quantitative relationships hold for this regulatory mechanism. It has been possible to demonstrate (FIGURE 12) that the degree of inhibition*

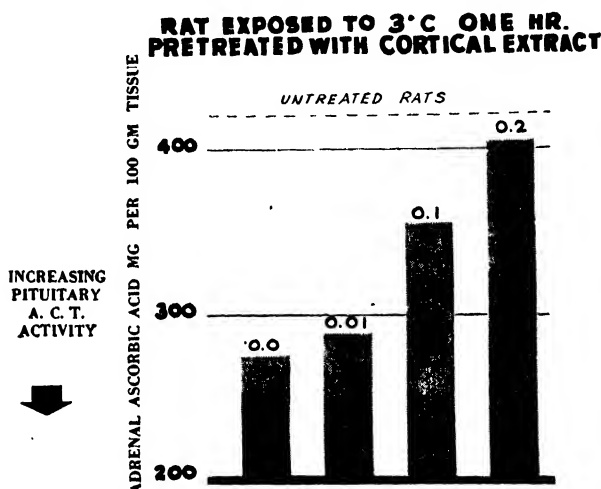


FIGURE 12. The relation between the degree of inhibition of pituitary adrenocorticotrophic activity and the dose of cortical hormone administered. The level of the dashed line represents the concentration of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of a column extends below the dashed line is proportional to the amount of A.C.T.H. elaborated by the pituitary. The figures atop the columns represent the milliliters of aqueous cortical extract (Upjohn) per 100 grams of body weight injected subcutaneously one hour before exposure to cold.

*of pituitary adrenocorticotrophic activity is proportional to the amount of administered cortical hormone.* As can be seen from FIGURE 12, 0.2 ml. of cortical extract per 100 gm. of body weight prevented the reduction in adrenal ascorbic acid which otherwise followed exposure of rats to 3°C. On the other hand, 0.01 ml. had very slight, if any, and 0.1 ml. had only partial blocking effect.

It has also been possible to demonstrate (FIGURE 13) that the *greater the degree of stress to which the animal is subjected the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotrophic activity.* Whereas 20 micrograms of 17-hydroxycorticosterone per 100 gm. of body weight completely inhibited the increase in adrenal cortical activity which otherwise follows injection of 0.25 mg. of histamine, this dose of steroid produced only partial inhibition in rats treated with 0.5 mg. of histamine.

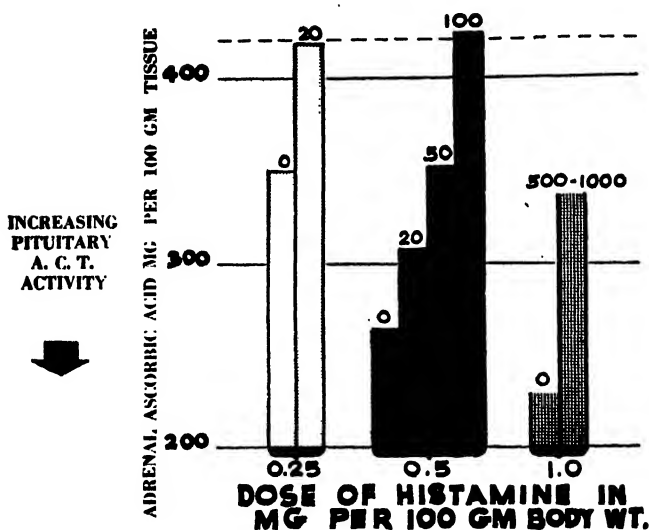


FIGURE 13. Relations between degree of stress and the dose of cortical hormone required to partially or completely inhibit pituitary adrenocorticotrophic activity. The level of the dashed line represents the concentration of ascorbic acid in the adrenals of untreated rats. The height of the columns represents the concentrations of ascorbic acid in the adrenals of treated rats. The distance the top of the column extends below the dashed line is proportional to the amount of A.C.T.H. elaborated by the pituitary. The figures atop the columns represent the amount of 17-hydroxycorticosterone in micrograms per 100 grams of body weight injected subcutaneously one hour before histamine administration.

The greater pituitary adrenocorticotrophic activity produced by this larger dose of histamine could be blocked by administration of 100 micrograms of 17-hydroxycorticosterone. In animals treated with 1.0 mg. of histamine, a dose of 1.0 mg. of 17-hydroxycorticosterone resulted in only partial suppression of pituitary activity. No more than a guess may be made at the present time as to the reason why even large doses of cortical hormone were unable to bring about complete inhibition of pituitary activity following the administration of 1.0 mg. of histamine per 100 gm. of body weight. At least two possibilities exist: (1) complete inhibition requires still larger doses than those used; (2) some factor, other than the rate of utilization of cortical hormone, comes into play in severe stress to excite the pituitary.

From these data, we may interpret the interrelationships among the pituitary, the adrenal cortex, and the tissue cells as diagrammatically presented in FIGURE 14. Under optimal conditions of the environment, the pituitary-adrenal system is in a relatively quiescent state. The peripheral tissue cells, under these conditions, require relatively small amounts of cortical hormone(s), and the concentration of the latter in the circulating blood is maintained at a level which holds the anterior pituitary in check. This means that, under optimal conditions of the environment, minimum amounts of A.C.T.H. are elaborated by the anterior pituitary and minimum amounts of cortical hormone(s) are secreted by the adrenal cortex.

This is to be contrasted with the activity of the pituitary-adrenal system which occurs under conditions of stress. The requirement for cortical hor-

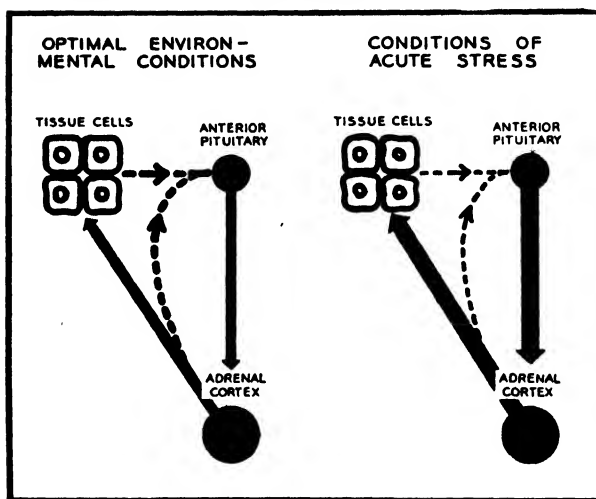


FIGURE 14. The pituitary-adrenal system under optimal environmental conditions and under conditions of stress. The width of the lines is proportional to the concentration of hormone in the body fluids. The two dashed lines, one off to the side of the tissue cells, and the other going directly from the tissue cells to the pituitary, indicate that the cortical hormones may have a direct inhibitory effect on the pituitary or that they may have an indirect effect through some product of their metabolic action on the tissue cells.

mone(s) by the tissue cells is accelerated and hence, the quantity in the circulating blood is reduced. The check on the anterior pituitary is now removed and A.C.T.H. is elaborated in relatively large quantities. Increased secretory activity of the adrenal cortex occurs, meeting the needs of the peripheral tissue cells for the cortical steroids. This increased activity of the pituitary-adrenal system continues until the stress is removed or until adaptation occurs, at which time, the requirement of the peripheral tissue cells for cortical hormone(s) is diminished.

The quantitative relationships which hold for this regulatory mechanism may be adequately explained if it is assumed that the rate of elaboration of A.C.T.H. from the pituitary can fluctuate by gradual change and according to the requirements of the tissue cells for cortical steroids. Just as the pituitary-thyroid system maintains the tissues in a state of well-being with regard to thyroid hormone, so the pituitary-adrenal system maintains the tissues in a state of well-being with regard to adrenal cortical hormone(s). The term, euthyroidism, has been used to denote a state of well-being of the tissues with regard to thyroid hormone.<sup>59</sup> "Eucorticism" would be a comparable term to apply to the adrenal cortical hormone(s).

*Relative pituitary inhibitory potency of crystalline steroids.* An estimate of the pituitary inhibitory potency of five crystalline steroids has been made. The approximate potency rating has been assigned on the basis of the comparative ability of these substances to block the pituitary adrenocorticotrophic activity which accompanies exposure to a uniform degree of cold or which follows injection of a standard dose of histamine. It is to be noted that the experiments were of short duration. A period of one hour elapsed between injection of the steroid and subjection of the animal to stress.

Hence, the estimated potencies of these steroids are undoubtedly influenced by their relative solubilities in the vehicle as compared to the tissue fluids surrounding the subcutaneous site of injection.

Corticosterone has been assigned a pituitary inhibitory potency rating of one (TABLE 1). Seventeen-hydroxycorticosterone and 17-hydroxy-11-

TABLE 1  
RELATION BETWEEN METABOLIC ACTIVITY AND PITUITARY INHIBITORY POTENCY OF FIVE CRYSTALLINE STEROIDS (SEE TEXT FOR EXPLANATION)

Steroid Hormone	a	b	c
	Sodium Retention	Glycogenesis	Pituitary Inhibition
17-Hydroxy-corticosterone	0	100	4
17-Hydroxy-11-Dehydro-corticosterone	0	90	4
Corticosterone	50	67	1
11-Desoxy-corticosterone	100	0	$\frac{1}{2}$
Progesterone	5†	0*	$\frac{1}{50}$

a. Ingle.<sup>21</sup>

b. Olson *et al.*<sup>51</sup>

\* Lacks diabetogenic and anti-insulin activities.<sup>64</sup>

† Thorn and Engel.<sup>70</sup>

dehydrocorticosterone are of equal potency and are about four times as active as corticosterone. Desoxycorticosterone, despite limitations in regard to solubility, is approximately one-half as potent as corticosterone, while progesterone has only  $\frac{1}{50}$  the activity of corticosterone. It is apparent that both types of cortical steroids, the C<sub>11</sub> oxygenated and the C<sub>11</sub> desoxy type, possess pituitary inhibitory potency. In TABLE 1, the relative potencies of the five steroids in regard to sodium retention and glycogenesis are also presented. The most potent compounds have been given a rating of 100. There is no parallelism between the effect of the steroids on electrolyte metabolism, carbohydrate metabolism, and their ability to inhibit pituitary adrenocorticotrophic activity. Desoxycorticosterone, 17-hydroxycorticosterone, and 17-hydroxy-11-dehydrocorticosterone all have pituitary inhibitory potency, but, whereas desoxycorticosterone produces sodium retention and has no glycogenetic activity, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone produce sodiumphoresis and are most potent in glycogenetic activity. It is of interest, in this connection, that Ingle and Kendall<sup>25</sup> came to the conclusion that the level of sodium or potassium in the body fluids does not directly influence pituitary adrenocorticotrophic activity.

If the "direct action" hypothesis is correct, then it necessarily follows that administration of desoxycorticosterone in suppressing pituitary adrenocorticotrophic activity should result in a deficiency of endogenously secreted C<sub>11</sub> oxygenated steroids. There is evidence to suggest that this is indeed true. Clinical reports indicate that DOCA increases the tolerance to glucose of diabetic patients.<sup>28, 29, 41, 77</sup> Selye *et al.*<sup>66</sup> have found that chronic treatment with DOCA produces adrenal cortical atrophy and im-

pairs the ability of the rat to mobilize glucose when subjected to a variety of non-specific types of stress.

Further investigation of the relation between metabolic activity, chemical structure, and pituitary inhibitory potency is being conducted at the present time. The data which have been obtained so far suggest, but do not offer conclusive proof, that cortical steroids have a direct action on the pituitary rather than an indirect one through some product of their metabolic activity or through their deficiency.

*Summary.* The administration of cortical hormone suppressed the enhanced pituitary adrenocorticotrophic activity which otherwise accompanies environmental change (heat, cold), drug administration (histamine, epinephrine), and bacterial intoxication (injection of a suspension of killed typhoid organisms). The data may be interpreted to mean that the great variety of non-specific stresses increases pituitary adrenocorticotrophic activity by a common mechanism, namely, by increasing the requirement of the peripheral tissue cells for cortical hormone(s). The hypophysis responds to the decrease in concentration of cortical hormone in the body fluids by increasing the rate of elaboration of trophic hormone.

Certain quantitative relationships hold for this regulatory mechanism. First, the degree of inhibition of pituitary adrenocorticotrophic activity is proportional to the amount of administered cortical hormone. Second, the greater the degree of stress to which the animal is subjected, the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotrophic activity. These quantitative data may be adequately explained if it is assumed that the rate of elaboration of A.C.T.H. from the pituitary can fluctuate by gradual change and according to the requirements of the tissue cells for cortical steroids. It is suggested that this regulatory mechanism maintains the tissue cells in a state of "eucorticism" during environmental change and exposure to moderate degrees of stress which the organism encounters in its everyday existence.

Since pituitary inhibitory potency resides in both the  $C_{11}$  oxygenated and the  $C_{11}$  desoxy type steroids, it is suggested, that the steroids act directly on the anterior pituitary rather than indirectly through products of their metabolic activity or their deficiency.

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# THE CHEMISTRY AND PARTIAL SYNTHESIS OF ADRENAL STEROIDS

By EDWARD C. KENDALL\*

*Division of Biochemistry, Mayo Foundation, Rochester, Minnesota*

It is just ten years since Steiger and Reichstein<sup>1</sup> prepared desoxycorticosterone by partial synthesis. It is fortunate that the yield of this steroid and the supply of starting material were sufficient to permit preparation of large amounts of this hormone so that its physiological activity could be determined both in experimental animals and in patients who had Addison's disease. These studies soon indicated that, although there was a marked effect on the metabolism of sodium, potassium, chloride, and water, desoxycorticosterone produced but little effect on the metabolism of fat, carbohydrate, and protein. It did not markedly increase the resistance of animals to stress, toxic compounds, and infections. There was a wide discrepancy between the physiological effect of extracts of the adrenal cortex and that of desoxycorticosterone.

At the same time, it was shown that the crystalline hormones of the adrenal cortex with an atom of oxygen at C<sub>11</sub>, in experimental animals, did influence the metabolism of fat, carbohydrate, and protein, and also increased the resistance to stress, toxic compounds, and infections. However, the amounts of material available were insufficient to extend these observations to a clinical study of patients who had Addison's disease.

In 1940, since it seemed probable that the hormones of the adrenal cortex would be useful therapeutic agents in the treatment of shock and trauma encountered in military operations, several laboratories throughout the world started investigations with the objective of preparing these hormones by partial synthesis. A survey of available starting material indicated that plant steroids could not readily be used, since oxygen could not be added at C<sub>11</sub>. However, the bile acids provided a source of steroids which theoretically could be rearranged with an atom of oxygen at C<sub>11</sub>.

Desoxycholic acid appeared to be a practical starting material, and experience has shown that this conclusion was correct. The conversion of desoxycholic acid to hormones of the adrenal cortex involves four changes, each one of which is composed of from two to twelve steps. These changes are: (1) introduction of oxygen at C<sub>11</sub>; (2) removal of the side chain at C<sub>20</sub>; (3) elaboration of the ketol group at C<sub>20</sub>:C<sub>21</sub>; and (4) formation of an unsaturated ketone in ring A.

The sequence of the steps which have been used in the conversion of desoxycholic acid to the hormones of the adrenal cortex is as follows: In the preparation of dehydrocorticosterone, Lardon and Reichstein<sup>2</sup> (1) removed the side chain; (2) introduced oxygen at C<sub>11</sub>; (3) elaborated the ketol group at C<sub>20</sub>:C<sub>21</sub>; and (4) formed a double bond ketone in ring A. Von Euw, Lardon, and Reichstein,<sup>3</sup> in the partial synthesis of corticosterone, (1) re-

\* This work has been made possible by the cooperation of the following: B. F. McKenzie, W. F. McGuckin, L. L. Engel, R. B. Turner, V. R. Mattox, and G. A. Fleisher.

moved the side chain; (2) introduced the hydroxyl group at  $C_{11}$ ; (3) elaborated a ketol group at  $C_{20}:C_{21}$ ; and (4) formed a double bond ketone in ring A. For the partial synthesis of 17-hydroxydehydrocorticosterone, Sarett<sup>4</sup> (1) removed the side chain; (2) introduced oxygen at  $C_{11}$ ; (3) elaborated the trihydroxy group  $C_{17}:C_{20}:C_{21}$ ; (4) formed the double bond ketone in ring A; and (5) converted the hydroxyl group at  $C_{20}$  to a ketone.

The partial synthesis of these three hormones furnished evidence for the structures previously assigned on the basis of their chemical properties, but the yields precluded the use of the original methods for the commercial preparation of these hormones. The only possibilities to make them available for use in clinical medicine are to increase the yield by the method of preparation originally used or to find better methods of preparation. It is my purpose, at this time, to report the progress which has been made in the partial synthesis of the hormones of the adrenal cortex as of today.

*Introduction of Oxygen at  $C_{11}$ .* In the early investigation, the problem of major importance was introduction of oxygen at  $C_{11}$ . Reichstein and his associates<sup>5, 6</sup> accomplished this by formation of the double bond  $C_{11}:C_{12}$  by pyrolysis of methyl 3-keto-12-benzoycholanate which was prepared from desoxycholic acid. Reduction of the ketone at  $C_3$  and addition of hypobromous acid followed by oxidation yielded the 11-keto-12-bromo compound. Reductive debromination gave the 11-keto derivative. There is a large amount of labor involved in this method for introduction of oxygen at  $C_{11}$ . Since this initial work, two other satisfactory methods have been devised.

Several years ago, Marker and Lawson,<sup>7</sup> and also Longwell and Wintersteiner,<sup>8</sup> investigated the replacement of the atom of bromine in methyl 3-acetoxy-11-bromo-12-ketocholanate. They prepared a dihydroxyketo compound which was designated as a 3( $\alpha$ ),11-dihydroxy-12-ketocholanic acid. Gallagher<sup>9</sup> prepared the ( $\alpha$ ) and ( $\beta$ )-11-monobromo derivatives of methyl 3( $\alpha$ )-acetoxy-12-keto-cholanate in crystalline form and from these prepared the 11( $\alpha$ ) and ( $\beta$ )-hydroxy-12-keto compounds. He then showed that if these compounds were boiled in alkaline solution a rearrangement of the ketol occurred with formation of the 11-keto-12-hydroxy compound in a yield of approximately 70 per cent. This investigation clarified the results of the early investigators. The 11-hydroxy compound is obtained under mild conditions when the atom of bromine is replaced with a hydroxyl group; but at a higher temperature, the rearrangement of the ketol readily occurs and the acid previously described as the 11-hydroxy-12-keto compound is, in fact, the 11-keto-12-hydroxy derivative. Subsequently, Borgstrom and Gallagher<sup>10</sup> showed that the hydroxyl group at  $C_{12}$  could be replaced through the action of phosphorus tribromide with formation of the 11-keto-12( $\alpha$ )-bromo derivative. Reductive debromination yielded the 11-keto compound.

The other method for the introduction of oxygen at  $C_{11}$  was devised in my laboratory.<sup>11</sup> The basis for this method is the replacement of the atom of bromine at  $C_{11}$  in an 11,12-dibromo derivative. It had previously been found that, when 3( $\alpha$ )-hydroxy-11,12-dibromoeholanic acid was heated with sodium hydroxide, a double bond was introduced at  $C_9:C_{11}$ , and the atom of bromine at 12 was replaced with the hydroxyl group.<sup>12, 13</sup> Subsequently, it

was found that, if the atom of hydrogen at 9 was replaced with a cyclic ether 3,9, then a double bond 9,11 could not be formed, and treatment of the 11( $\beta$ ),12( $\alpha$ )-dibromo compound resulted in an almost quantitative substitution of bromine at C<sub>11</sub> with oxygen. The 3,9-cyclic ether was subsequently opened with hydrogen bromide, and methyl 3( $\alpha$ )-acetoxy-11-keto-12( $\alpha$ )-bromocholanate was separated in good yield. The interesting feature of this method for introduction of oxygen at C<sub>11</sub> is the surprisingly high yields for the intermediate compounds. The details of these reactions have been published and will not be repeated in this paper.<sup>11, 13-17</sup>

Since the series of reactions was first devised, this method has been found satisfactory for the preparation of large amounts of steroids with oxygen at C<sub>11</sub>.

*Removal of the Side Chain.* Until recently, the most satisfactory method for removing the side chain at C<sub>20</sub> was through the stepwise degradation devised by Barbier and Locquin<sup>18</sup> and adapted to the steroids by Wieland and his associates.<sup>19</sup> During work on this phase of the problem in my laboratory, significant improvements were made in the use of this method.<sup>11</sup> The usual yield in the past for the formation of the diphenylcarbinol has been 50 to 60 per cent. Dehydration of the carbinol to yield a diphenylethylene was carried out without significant loss, but the oxidation of the diphenylethylene to the nor acid usually gave yields of the order of 50 to 60 per cent.

At a low temperature, from 0° to 5°, it was found that the methyl 3,9-epoxy-11-keto-12-bromocholanate formed the diphenylcarbinol in a yield of more than 90 per cent. The atom of bromine at C<sub>12</sub> adjacent to the ketone was simultaneously removed<sup>20</sup> through the action of the Grignard reagent. This formed a convenient method to prepare the debrominated product without the need of an extra step. The yield of the diphenylethylene from the carbinol was practically quantitative, and the oxidation of the diphenylethylene to the nor acid was accomplished in a yield of more than 90 per cent. In other words, from the cholanic acid to the nor acid the yield was raised to approximately 80 per cent. The degradation of the second atom of carbon to give the bisnor acid was carried out in comparable yields, and the conversion of the bisnor ester to the ethylene with double bond C<sub>20</sub>:C<sub>22</sub> was accomplished in a yield of approximately 75 per cent.

Within the past three years, Miescher and associates have described a new method to shorten the labor involved for the degradation of the side chain at C<sub>20</sub>. Ziegler and associates<sup>21</sup> previously had studied bromination of unsaturated compounds with bromosuccinimide. When bromination of the diphenylethylene was attempted by the use of bromosuccinimide, the yields were too low to compete with the degradation through the Barbier-Wieland procedure. Recently, in Miescher's<sup>22</sup> laboratory, it was found that good yields of the 22-monobromo derivative of the diphenylethylene could be obtained if the bromination was carried out in the presence of light. Subsequent dehydrobromination gave the diene  $\Delta^{20, 22}$ ; <sup>23, 24</sup> Through this procedure, in two steps which were carried out in a single operation, a double bond was introduced at C<sub>20</sub>:C<sub>22</sub> and a single oxidation furnished the ketone at C<sub>20</sub>. However, with the compounds under consideration, the

over-all yield of the 20-ketone through this method was far from quantitative. To the present, the best yield has been in the neighborhood of 38 per cent. Since 34 per cent could be obtained through the stepwise degradation, there is not much advantage in the use of the diene, except that the amount of labor involved is much less.

*Elaboration of the Ketol.* For elaboration of the ketol group at  $C_{20}:C_{21}$ , Reichstein and his associates have used two methods. In the preparation of dehydrocorticosterone<sup>2</sup> the 20-ketone with a methyl group at  $C_{21}$  was converted to the etio acid by removal of the 21-methyl group. Through the acid chloride of the etio acid thus obtained, diazomethane afforded the diazoketone, which was, in turn, converted to the 21-acetoxy, 20-ketone compound. The other method was the direct formation of the 21-acetoxy group from 3,11-dihydroxy-20-ketopregnane.<sup>3</sup> This was accomplished with lead tetracetate.

After Miescher and associates had devised a method to form the  $\Delta^{20, 22; 23, 24}$  derivative, it appeared possible from previous work in my laboratory that further bromination would yield either the  $C_{21}$ -bromo or  $C_{17}$ -bromo compound. In either case, the product could be used to advantage. Bromination of 3( $\alpha$ )-acetoxy-11-keto-12-bromo-24,24-diphenyl- $\Delta^{20, 22; 23, 24}$ -choladiene with bromosuccinimide was therefore tried, and it was found that bromine substituted hydrogen at  $C_{21}$ . This 12,21-dibromo compound of the diene separated in pure form in a yield of approximately 62 per cent. This is in contrast to the preparation of the  $\Delta^{20, 22; 23, 34}$ -diene from 3( $\alpha$ )-acetoxy-11-keto-12-bromo-24,24-diphenyl- $\Delta^{23, 24}$ -cholene. These two last-mentioned compounds form a mixture which cannot readily be separated. All attempts to prepare a pure sample of the  $\Delta^{20, 22; 23, 24}$ -diene have been unsuccessful. Neither crystallization from various solvents, nor chromatography, has yielded a pure sample of the diene. However, the introduction of the bromine at  $C_{21}$  modified the ease of crystallization and the 12,21-dibromodiene separated readily.

It was also found that the atom of bromine at  $C_{21}$  in methyl 3( $\alpha$ )-acetoxy-11-keto-12,21-dibromo-24,24-diphenyl- $\Delta^{20, 22; 23, 24}$ -choladiene is replaced very easily either with the acetoxy or with the methoxy group. The 21-acetoxy and 21-methoxy compounds have been separated in pure crystalline form. Oxidation with chromic acid gave the 20-ketone with the acetoxy or methoxy group at  $C_{21}$ . It has also been found that in methanol the 21-bromodiene, with an acetoxy group at  $C_3$ , was converted into the 3( $\alpha$ )-hydroxy-21-methoxy compound. This, in turn, with hydrogen bromide, was converted in excellent yield to the 21-bromo-3( $\alpha$ )-hydroxy derivative. With acetic acid and sodium acetate the 21-acetoxy group was restored, and a single oxidation with chromic acid then yielded the 3,11,20-triketo-21-acetoxy compound.

It should be noted that the introduction of oxygen at  $C_{11}$  as the first step and subsequent elaboration of the ketol at  $C_{20}:C_{21}$  from the choladiene derivative completes three of the four changes with a minimal amount of labor, and the excellent yields indicate that it will be difficult to secure significant improvement for these three steps.

*The Unsaturated Ketone in Ring A.* For the partial synthesis of the hormones of the adrenal cortex without oxygen at  $C_{11}$ , desoxycorticosterone, steroids such as cholesterol, stigmasterol, or other starting material with a double bond  $C_5:C_6$  have been used. To change the double bond from its original position to  $C_4:C_5$ , it is necessary only to change the 3-hydroxyl group to a ketone. The double bond then shifts to form a conjugated group with the ketone in the A ring.<sup>23</sup> In the partial synthesis of the hormones with oxygen at  $C_{11}$ , it is necessary to start with a saturated bile acid and to introduce the double bond either in the A or in the B ring. Experience has shown that the best sequence for introduction of the double bond ketone in the A ring is after introduction of oxygen at  $C_{11}$  and elaboration of the ketol at  $C_{20}:C_{21}$ .

The simplest method to introduce the double bond is to substitute an atom of hydrogen at  $C_4$  with bromine and then to remove hydrogen bromide.<sup>24</sup> Bromination at  $C_4$  can be carried out with satisfactory yields, but unfortunately, removal of hydrogen bromide has not been accomplished with a correspondingly good yield.<sup>2</sup> At the present moment, this step in the partial synthesis is attended with loss of material which is greater than the loss at any other of the many steps. It seems highly probable that this modification in the structure of the steroid eventually will be accomplished in yields which are substantially higher than those which have been obtained in the past.

*Introduction of Hydroxyl Group at  $C_{17}$ .* Corticosterone and dehydrocorticosterone are examples of hormones of the adrenal cortex which have the ketol group at  $C_{20}:C_{21}$  and oxygen at  $C_{11}$ . During the past several years, evidence has accumulated that the physiologic action of corticosterone and dehydrocorticosterone is quantitatively inferior to that of the corresponding hormones which have in addition a hydroxyl group at  $C_{17}$ .

For the partial synthesis of hormones with a hydroxyl group at  $C_{17}$ , but without oxygen at  $C_{11}$ , von Euw and Reichstein<sup>25</sup> have prepared a 17,20,21,22-tetrahydroxy compound. The 21,22-acetone derivative of this was acetylated at 20 and, after removal of the acetone moiety, the 20,21-glycol was then converted with periodic acid to the 20-acetoxyl-21-aldehyde compound. Removal of the 20-acetyl group and treatment with pyridine brought about a rearrangement of the 20-hydroxy-21-aldehyde to the 21-hydroxy-20-ketone structure.

For the partial synthesis of 17-hydroxydehydrocorticosterone, Sarett<sup>4</sup> has devised a method which has as its essential step the preparation of a 17,20-dihydroxy-21-acetoxyl derivative through hydroxylation of 3( $\alpha$ ),21-diace-toxy-11-keto- $\Delta^{17,20}$ -pregnene. Oxidation with chromic acid yielded the 20-keto compound, but the yields of the intermediate compounds preclude the use of this method of synthesis for large-scale production.

The hormone with the remaining possible structure, that is, 17-hydroxy-corticosterone (compound F), has not yet been partially synthesized.

The success which has attended efforts in the past is the best assurance that the remaining rough spots will be smoothed out and satisfactory methods for the commercial preparation of all of the hormones of the adrenal cortex will be devised.

It is interesting to note the progress which has been made since the partial synthesis of dehydrocorticosterone in 1943. The method of Lardon and Reichstein<sup>2</sup> required forty-one steps; the yield was approximately 0.04 per cent. Introduction of oxygen at C<sub>11</sub> through the 3,9-epoxy compound, removal of the side chain, and elaboration of the ketol at C<sub>20</sub>:C<sub>21</sub> through the diene reduced the number of steps to twenty-six and increased the yield, up to the introduction of the double bond C<sub>4</sub>:C<sub>5</sub>, to about 10 per cent. At the present time, the introduction of the double bond C<sub>4</sub>:C<sub>5</sub> lowers the yield to between 3 and 4 per cent of that theoretically possible. Even with this great loss at the last step the improvement from the first synthesis is indeed striking.

*Physiological Effects of the Synthetic Compounds.* With the hormones at hand, what may be expected from their use in clinical medicine? I shall not venture a prediction, but shall point out that the critical condition of patients who have severe insufficiency of the hormones of the adrenal cortex can be controlled, if adequate amounts of the hormones are available. Furthermore, there is good evidence that under stress the adrenal cortex is stimulated to pour out its secretion. It does not seem probable that all patients are able to produce sufficient of the secretion at all times. If the hormones become plentiful enough to permit study of patients under conditions of stress, it may be possible to develop clinical tests to show that a deficiency exists and to treat patients who have latent or actual insufficiency.

About a year ago, Merck & Co., Inc., prepared a large sample of dehydrocorticosterone by the method which I have outlined for introduction of oxygen at C<sub>11</sub> through the 3,9-epoxy structure. With experimental animals, it was shown by Drs. Dorfman, Venning, and Lewis that the crystalline hormone prepared by partial synthesis was identical with the natural product.\* The identity of the chemical structure in the natural and partially synthesized products was beyond doubt. However, it was a matter of no little interest and importance to prove quantitatively that the biologic response to the two different preparations was the same.

Dehydrocorticosterone prepared by partial synthesis was used in the treatment of a series of patients who had Addison's disease.<sup>26, 27</sup> The results, however, were not striking. The reason for the failure to respond may be that there are differences between the experimental animal and the human being. The effect in rats on deposition of glycogen in the liver showed less than a one-fold difference when dehydrocorticosterone was used in place of 17-hydroxydehydrocorticosterone (compound E<sup>28</sup>). The protection of animals with compound A against cold and against the toxic effects of typhoid vaccine were notable.† However, in the patients who had Addison's disease, continued administration of large amounts of compound A did not produce a marked effect.<sup>29</sup>

It has already been shown that dehydrocorticosterone is much less toxic to normal mice than is compound E. To show how the mouse can tolerate compound A and how impossible it is to predict the physiologic effects of large amounts of these hormones, I shall report briefly some results obtained

\* These results were reported at a symposium held in Atlantic City, New Jersey, March 11, 1946.

† These results are given in the reports of Drs. Dorfman, Venning and Lewis which follow.

by Dr. Heilman and myself.<sup>30</sup> Pellets of crystalline compounds A, B, and E were given to four strains of mice over a period of four to five weeks. Under the conditions used, compound A did not cause death or lesions in the muscles. However, surprisingly large amounts of fat were deposited, even in young mice only 10 gm. in body weight. There was a slow increase of body weight with compound A. With compound B, there was a loss of weight, but still a marked increase of the deposition of fat. With compound E, there was more serious loss of body weight, and no deposition of fat and lesions were present in the muscles. The significance of these observations in the interpretation of the symptoms of patients who have hyperactivity of the adrenal cortex remains to be shown. It seems probable, however, that loss of muscle and deposition of fat in Cushing's disease may be related to the overproduction of compounds A, B, E, and F.

My chief interest for the past six years in the problems associated with the adrenal cortex has been to make the active compounds available for use. In this phase of the problem, it is a matter of satisfaction to be able to report the progress which has been made through the use of synthetic organic chemistry as a tool for the investigation of problems in normal physiology and clinical medicine.

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## APPENDIX

(The following three papers constitute the first demonstration that the biological properties of synthetic and natural 11-dehydrocorticosterone are identical.)

### 1. STUDIES ON THE PROTECTIVE POWER OF ADRENAL EXTRACT AND STEROIDS AGAINST BACTERIAL TOXINS IN ADRENALECTOMIZED RATS

By LENA A. LEWIS AND IRVINE H. PAGE

*Research Division of the Cleveland Clinic Foundation, Cleveland, Ohio*

It has been shown that the protective power of adrenal extracts or steroids against the acute toxic effect of typhoid vaccine can be accurately measured by testing the material on adrenalectomized rats.<sup>1</sup>



The assay is carried out on male Sprague-Dawley rats weighing between 80 and 120 gms., which are adrenalectomized under ether anesthesia, and maintained on 0.9 per cent sodium chloride drinking water for at least 5 days before starting the test. The animals are injected subcutaneously, once daily, if the hormone is in oil solution, twice daily, if in aqueous or alcohol-aqueous solution. Immediately after the third morning injection of hormone, 1.33 minimal lethal doses (M.L.D.) of typhoid vaccine are administered intraperitoneally. The M.L.D. is determined previously on saline-maintained adrenalectomized rats. The percentage of rats surviving 24 hours after receiving 1.33 M.L.D. typhoid vaccine is noted. One toxic protection unit, as we have defined it, is the amount of material, in terms of any convenient unit, per 24 hours, which will protect 90 per cent of adrenalectomized rats against 1.33 M.L.D. of typhoid vaccine and is equivalent in protective power to 0.28 mg. 11-dehydrocorticosterone acetate.

A series of experiments were carried out using 11-dehydrocorticosterone (Compound A) and 11-dehydrocorticosterone acetate prepared by isolation from adrenal tissue, 11-dehydrocorticosterone acetate prepared by partial synthesis,<sup>3</sup> and Compound E. The results obtained on the 4 materials are summarized in FIGURE 1. The natural and synthetic Compound A acetate,

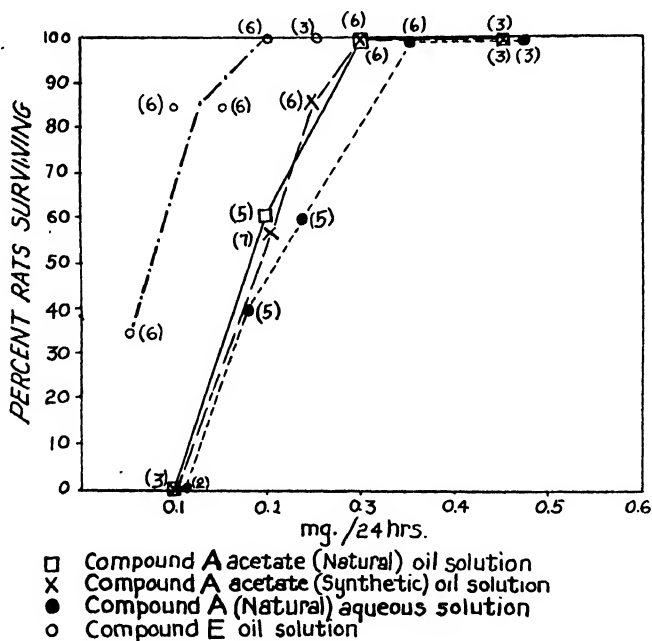


FIGURE 1. Comparison of protective power of natural and synthetic Compound A Acetate, Compound A, and Compound E against typhoid vaccine in adrenalectomized rats.

when administered in oil, gave nearly identical results. The natural Compound A in aqueous solution appeared to be slightly less effective. Ninety per cent of the rats were protected by 0.28 mg. of the Compound A acetate. Compound E was the most active of the materials tested.

Blood leucocyte counts and differential blood counts were made on adrenalectomized rats which were maintained on saline, or treated with whole adrenal extract, or with synthetic Compound A acetate before, and 2 hours after, the intraperitoneal injection of 1.33 M.L.D. of typhoid vaccine. TABLE 1 summarizes the results obtained. In the extract-treated and Com-

TABLE 1  
ADRENALECTOMIZED RATS

	W.B.C. 1000/ cmm.	Per cent lympho- cytes	Lym- pho- cytes 1000/ cmm.	Per cent neutro- philes	Neutro- philes 1000/ cmm.
<i>Extract treated</i>					
<i>Before typhoid</i>					
Average	28.9	77.6	22.4	19.4	5.8
13 animals range	(23.25-41.75)	(69-88)		(8-29)	
<i>Two hours after typhoid</i>					
Average	29.7	49.2	14.6	46.2	13.7
13 animals range	(22.0-42.0)	(29-67)		(31-69)	
Change	+0.8		-7.8		+7.9
<i>Saline treated</i>					
<i>Before typhoid</i>					
10 animals	31.7	81.4	25.8	15.2	4.8
	(22.9-50.5)	(72.88)		(6-24)	
<i>Two hours after typhoid</i>					
	27.5	64.4	17.7	30.0	8.4
	(20.1-36.8)	(45-87)		(7-48)	
Change	-4.2		-8.1		+3.4
<i>Compound A treated</i>					
<i>Before typhoid</i>					
15 animals	26.58	73.8	19.6	26.3	7.0
	(18.1-31.6)	(53-93)		(7-45)	
<i>Two hours after typhoid</i>					
	26.35	40.7	10.7	58.1	15.3
		(21-61)		(28-72)	
Change	+1.3		-8.9		+8.3
<i>Saline treated</i>					
<i>Before typhoid</i>					
13 animals	29.8	79.9	23.8	18.8	5.6
	(17.3-46.1)	(59-89)		(9-40)	
<i>Two hours after typhoid</i>					
	22.2	54.4	12.1	44.4	9.8
	(13.7-31.2)	(38-73)		(26-56)	
Change	-7.6		-11.7		+4.2

pound A acetate-treated rats, the total leucocyte count showed no significant change 2 hours following administration of toxin. The lymphocytes decreased 7.8 thousand per cu. mm. and 8.9 thousand per cu. mm. respectively,

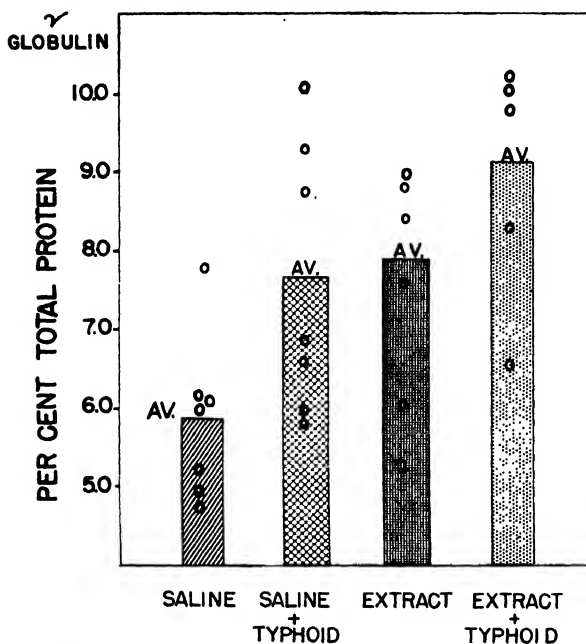


FIGURE 2. The  $\gamma$ -globulin concentration of serum of adrenalectomized rats treated with saline, or with adrenal extract before and 2 hours after administration of typhoid vaccine.

while the polymorphonuclear neutrophils increased 7.9 and 8.3 thousand per cu. mm. respectively. In contrast with the hormone-treated groups, the animals receiving no replacement therapy, except saline, showed a decrease in the total leucocyte count. The lymphocyte count decreased to about the same extent as that of the other groups, but the neutrophils showed a very much smaller increase. It is interesting that the changes in the blood picture are similar, whether the animal is treated with natural hormones or with synthetic Compound A acetate.

Whole adrenal extract and compound A acetate, also, have power to protect adrenalectomized rats against lethal doses of diphtheria toxin. Relatively larger amounts of hormone are required to protect against 1.25 M.L.D. of diphtheria toxin than against 1.33 M.L.D. typhoid toxin.

While the protective power of the various hormone preparations tested against typhoid toxin closely parallels their effect on carbohydrate metabolism, the maintenance of a normal blood sugar level is not alone capable of protecting the rats against the toxin.<sup>1</sup>

In an attempt to elucidate further the mechanism of the protective action of adrenal extract and steroids against typhoid toxin, serum proteins of adrenalectomized rats maintained on saline and of animals treated with whole adrenal extract were studied by the Tiselius electrophoresis method. Determinations were made on other similarly prepared animals 2 hours following the intraperitoneal injection of 1.33 M.L.D. typhoid vaccine. The average  $\gamma$ -globulin level of the extract-treated group was somewhat higher than the saline-treated animals, being 5.9 and 8.0 per cent respectively.

Following typhoid vaccine, the percentages increased in both groups, being 7.7 and 9.1 per cent respectively, 2 hours after administration of toxin. The increase in the  $\gamma$ -globulin is of interest in view of the fall in the lymphocyte counts. It has been shown by Dougherty and White<sup>2</sup> that the dissolution of lymphocytes is accompanied by an increase in  $\gamma$ -globulin of the serum.

### *Summary*

The toxic protective power in adrenalectomized rats of natural Compound A acetate and of Compound A acetate prepared by partial synthesis against 1.33 M.L.D. typhoid vaccine is identical. Compound A acetate and adrenal extract-treated adrenalectomized rats show a greater increase in blood polymorphonuclearneutrophils 2 hours following typhoid vaccine administration than do saline-treated controls 2 hours after toxin. A similar decrease in blood lymphocytes is observed in all groups.

The serum  $\gamma$ -globulin of extract-treated adrenalectomized rats is somewhat higher than that of saline-treated animals. Both groups show an increase in the  $\gamma$ -globulin level 2 hours after typhoid toxin administration.

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## 2. THE COMPARATIVE ACTIVITIES OF 11-DEHYDROCORTICOSTERONE ISOLATED FROM THE ADRENAL GLAND AND THAT PRODUCED SYNTHETICALLY

By RALPH I. DORFMAN

*Departments of Biochemistry and Medicine, Western Reserve University School of Medicine, and Lakeside Hospital, Cleveland, Ohio*

This study is concerned with the biological evaluation of the adrenal cortical steroid 11-dehydrocorticosterone isolated from the gland and also realized by partial synthesis.<sup>1</sup> Since the synthetic material available at the time of this study was in the form of the acetate, the direct comparisons were made on this derivative, rather than on the free compound. The comparisons were made using two biological tests, the deposition of glycogen in the adrenalectomized mouse<sup>2</sup> and the increase in survival of the adrenalectomized rat subjected to a low environmental temperature.<sup>3</sup> A further comparison is reported of the relative activity of the free compound on the basis of the deposition of glycogen to that of the acetate.

The methods used are those reported in detail previously from this laboratory.

*Comparison Using the Cold Test.* TABLE 1 summarizes the results on the cold test. Both the compound from the gland and that from the partial synthesis were run simultaneously to increase the precision of the comparison. The material was administered at four different concentrations ranging from 40 micrograms to 320 micrograms. Calculation of the best fitting

TABLE 1  
THE COMPARATIVE COLD TEST RESPONSE TO 11-DEHYDROCORTICOSTERONE  
ACETATE ISOLATED FROM THE ADRENAL GLAND AND THAT PRODUCED  
SYNTHETICALLY

Source of material	Amount administered	Number of adrenalectomized rats	Survival hours $\pm$ S.E.
0	$\mu\text{g.}$ 0	21	$9.4 \pm 0.37$
Synthetic	40	10	$10.7 \pm 0.88$
	80	10	$11.9 \pm 0.97$
	160	10	$13.5 \pm 1.17$
	320	10	$15.4 \pm 0.74$
Gland	40	10	$9.8 \pm 0.81$
	80	10	$11.3 \pm 1.23$
	160	10	$11.9 \pm 0.90$
	320	11	$14.2 \pm 0.98$

equations from the experimental data by the method of Fischer gave for the material from the gland

$$Y = 4.6x + 2.4,$$

and for the material from the synthesis

$$Y = 5.2x + 2.2.$$

The slopes of the curves were found not to be significantly different. The relative potency may be expressed as;

$$\frac{\text{Synthetic}}{\text{Gland}} = \frac{100}{60}.$$

At  $P = 0.05$  the error range was from +85 to -46 per cent. Therefore, the difference was not significant.

*Comparison Using Glycogen Deposition in the Rat.* TABLE 2 summarizes the results on the glycogen deposition in the adrenalectomized mouse. The 11-dehydrocorticosterone acetate obtained from the gland was studied at three concentrations ranging from 50 to 200 micrograms, while the synthetic material was studied at four concentrations ranging from 50 to 400 micrograms per animal. The material isolated from the gland gave the following equation as calculated from the experimental data:

$$Y = 3.95x - 5.4.$$

The synthetic material gave the following equation:

$$Y = 7.00x - 10.7.$$

The slopes of the two experimentally derived lines did not differ significantly. The relative potency of the glandular and synthetic materials is expressed by the following relationship:

$$\frac{\text{Synthetic}}{\text{Gland}} = \frac{100}{66}.$$

TABLE 2

THE COMPARATIVE GLYCOGEN RESPONSE TO 11-DEHYDROCORTICOSTERONE ACETATE ISOLATED FROM THE GLAND AND THAT PRODUCED SYNTHETICALLY

Source of material	Amount administered	Number of animals	Mg. of glycogen per 10g. of B.W. $\pm$ S.E.
0	$\mu$ g. 0	0	0.5 $\pm$ 0.2
Synthetic	50	7	1.1 $\pm$ 0.8
	100	14	3.3 $\pm$ 0.8
	200	12	5.6 $\pm$ 0.8
	400	14	7.4 $\pm$ 0.5
Glandular	50	8	1.0 $\pm$ 0.5
	100	13	2.8 $\pm$ 0.9
	200	14	3.5 $\pm$ 0.5

Calculation of the error of the potency ratio gave the limits at  $P = 0.05$  of  $+43$  to  $-30$  per cent.

The 11-dehydrocorticosterone was compared to that of the acetate by using the data previously reported for the free compound. On a molar basis, the following relative activity of the free compound and acetate was found to be:

$$\frac{\text{Free Compound}}{\text{Acetate}} = \frac{100}{63}$$

The error range of the potency ratio was found to be  $+29$  to  $-22$  per cent.

Thus, there appears to be no difference between the material isolated from the gland and that prepared by partial synthesis, when tested by either the cold test or by a method involving the carbohydrate activity. On a molar basis, the free compound appears to be more active than the acetate when tested by a glycogen deposition method in the mouse.

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### 3. BIOLOGICAL ACTIVITY OF SYNTHETIC 11-DEHYDROCORTICOSTERONE ACETATE

By ELEANOR H. VENNING

*McGill University Clinic, Royal Victoria Hospital, Montreal, Canada*

The biological activity of 11-dehydrocorticosterone acetate prepared by partial synthesis<sup>1</sup> was determined by the bioassay method of Venning, Kazmin, and Bell.<sup>2</sup> This method is based upon the ability of adrenal steroids to cause an increase in liver glycogen in fasted adrenalectomized mice. The animals are fasted for 16 hours prior to the assay, and the ex-

tract to be tested is administered in 7 divided doses, over a period of  $5\frac{1}{2}$  hours. Ten mg. glucose are given with each injection. This small amount of glucose fails to cause any significant increase in the liver glycogen of the control adrenalectomized mice, but has the effect of greatly increasing the sensitivity of the method. One hour following the last injection, the livers are quickly removed and the glycogen content determined. The amount of liver glycogen is compared with that deposited by a standard adrenal cortical compound.

The biological activity of the synthetic preparation has been compared with the natural 11-dehydrocorticosterone acetate derived from the gland and also with the free crystalline compounds, 11-dehydrocorticosterone and 17-hydroxy 11-dehydrocorticosterone.

The compounds were assayed at two dose levels, 40  $\mu$ g. and 80  $\mu$ g. respectively. Duplicate assays were carried out using a total of 8-9 mice per assay. In FIGURE 1, the logarithm dose-response regression lines of the

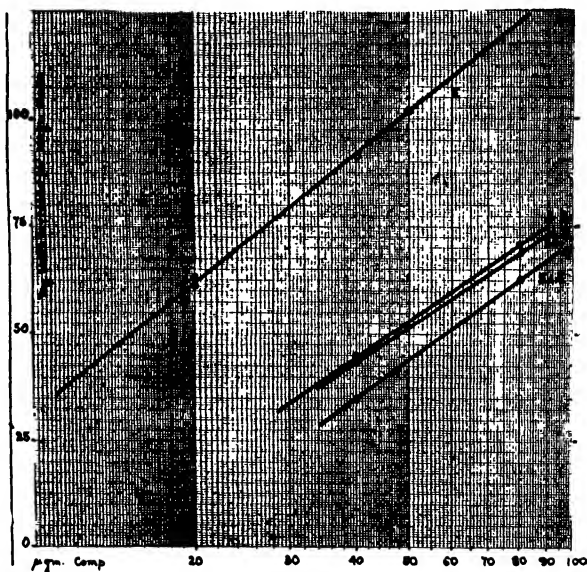


FIGURE 1. Logarithm dose-response regression lines. (1) 17-OH 11-dehydrocorticosterone, (2) 11-dehydrocorticosterone (3) 11-dehydrocorticosterone acetate (4) synthetic 11-dehydrocorticosterone acetate.

various compounds are shown. At a dose level of 80  $\mu$ g., synthetic 11-dehydrocorticosterone acetate caused a liver glycogen deposition of 63 mg. per 100 gm. body weight, 11-dehydrocorticosterone acetate (natural product from the adrenal gland), a deposition of 69 mg. per 100 gm. body weight, and the free compound 11-dehydrocorticosterone, a deposition of 70 mg. per 100 gm. body weight. Thus, the capacity of the synthetic compound to cause a deposition of liver glycogen is only slightly lower than that of the acetate derived from the adrenal gland. Acetylation of 11-dehydrocorticosterone apparently does not interfere with the biological activity of the hormone.

When the activity of 11-dehydrocorticosterone was compared with that of 17-hydroxy 11-dehydrocorticosterone, it was found that only 24  $\mu$ g. of the latter compound were required to cause a deposition of liver glycogen of 70 mg. per 100 gm. body weight. By this method of assay, 17-hydroxy-11-dehydrocorticosterone is over three times more active in its effect on liver glycogen deposition than 11-dehydrocorticosterone.

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# THE BIOASSAY OF ADRENAL CORTICAL STEROIDS

By RALPH I. DORFMAN

*Departments of Medicine and Biochemistry, Western Reserve University School of Medicine,  
and Lakeside Hospital, Cleveland, Ohio*

This paper is concerned with the evaluation of methods for the quantitative assay of the adrenal cortical activity of pure compounds, glandular extracts and urinary extracts. A relatively large number of methods have been suggested for this purpose, but only few have been studied in detail, and even fewer have been submitted to statistical analysis to ascertain the errors of estimation. It is the purpose of this summary to deal critically with representative methods which have been used. The methods will be discussed from the standpoint of sensitivity and reproducibility. No attempt will be made to evaluate all the variables in any one test; these details can be found in the original papers.

As a preliminary to the discussion of the methods for the assay of adrenal cortical material, it may be well to deal briefly with four topics which will help to define our problem. These topics are: (1) the role of the adrenal cortex in the body economy; (2) the biologically active substances or hormones which have been isolated from the adrenal cortex; (3) a summary of the various types of bioassays that have been suggested; and (4) a brief description of the statistical methods employed for data analysis.

## *Role of the Adrenal Cortex*

The physiological action of the adrenal cortical hormones has been excellently summarized by Ingle.<sup>11</sup> It is sufficient, for the purposes of this paper, to set forth some of the more important biological actions of the adrenal cortical hormones. The bulk of our knowledge of the biological action of these hormones has come from studies involving the removal of the adrenal cortex either from experimental animals or from the Addison's disease patient. Animals deprived of adrenal cortical hormones usually die in varying lengths of time, depending upon the species and age of the animal. The diet, particularly the concentrations of sodium and potassium, determine the survival time of the operated animals. Adrenalectomized animals are sensitive to all forms of stress such as changes in environmental temperature, external pressure, toxins, chemical poisons, infections, etc.

Adrenalectomy results in certain digestive disturbances such as loss of appetite, poor gastro-intestinal absorption, and diarrhea. The last symptom is particularly marked in advanced adrenal insufficiency.

Specific alterations in carbohydrate and mineral metabolism have been observed. Thus, in the absence of the adrenal cortex the animal appears to have difficulty in converting proteins to carbohydrates and tends to excrete excessive quantities of sodium and decreased quantities of potassium.

White and Dougherty<sup>20</sup> have demonstrated an influence of adrenal cortical steroids on protein metabolism. Essentially these workers have demonstrated that adrenal cortical hormones cause dissolution of lymphoid tissue,

a decrease in the total leucocyte count, a decrease in the absolute numbers of lymphocytes, and an increase in the absolute number of polymorphonuclear cells. Simultaneously, there is a rise in the total serum protein. The increase in plasma proteins appears in the globulin fraction.

Other changes in the adrenalectomized animal include such circulatory changes as hemoconcentration, decreased blood pressure, and blood flow.

*Hormones Isolated from the Gland.* All the symptoms which result from adrenal cortical insufficiency may be reversed by the administration of suitable extracts of the adrenal cortex. From these extracts, six well-defined compounds having adrenal cortical activity have been isolated and characterized.<sup>17</sup> Some of these compounds appear to be capable of correcting all of the symptoms, while others have more limited biological activity. The six compounds are illustrated in FIGURE 1. Four of the compounds,

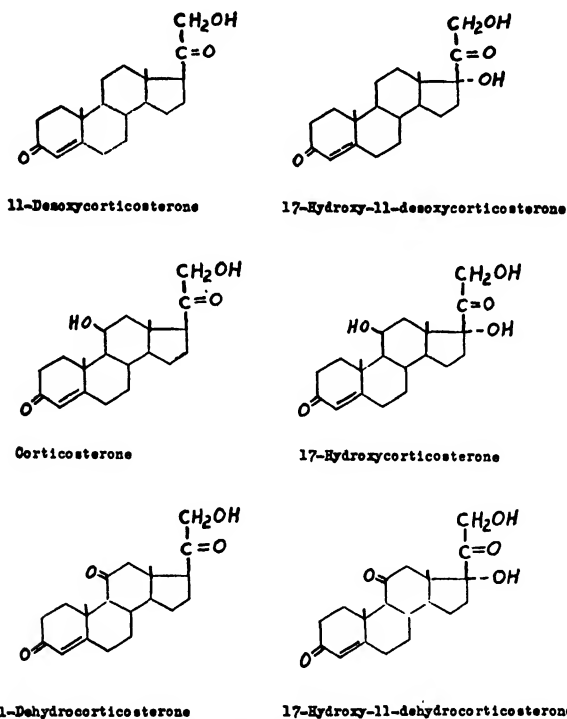


FIGURE 1. Steroids isolated from the adrenal cortex possessing biological activity.

corticosterone, 17-hydroxycorticosterone, 11-dehydrocorticosterone, and 17-hydroxy-11-dehydrocorticosterone, are characterized as having oxygen on carbon atom 11, either as an hydroxyl group or as a carbonyl group, while two of the compounds do not have oxygen at carbon 11. This is of interest, particularly, from the standpoint of carbohydrate metabolism, since the oxygen at carbon 11 appears to be essential for this activity. This point will be more clearly demonstrated later when we discuss the relative activi-

ties of the pure compounds. In addition to the six compounds mentioned, some twenty-odd other crystalline steroids have been isolated and identified. Some of these compounds have been shown to be inactive while others have not been adequately studied.

*Suggested Methods for Bioassay of Adrenal Cortical Hormones.*

The bioassay methods suggested in adrenalectomized animals may be classified as those dealing with growth and survival, those dealing with glycogen deposition in the liver of fasting animals or related effects on carbohydrate metabolism, and those dealing with various forms of stress and miscellaneous bioassays. It is fully realized that in some instances there exists overlapping as to type of test represented, and the classification used here is merely one of convenience.

*Survival-Growth Methods.* The adrenalectomized dog, cat, rat, mouse, drake, and guinea pig have been employed from time to time as test animals. Such end points as growth of the adrenalectomized animal, the length of survival, or percentage survival have been employed. One important factor is diet, since a diet rich in sodium and poor in potassium is known to lead to indefinite survivals in the absence of undue stress or infections. Some preliminary evaluations of a growth-survival method using the rat will be presented.

Bülbring<sup>1</sup> described a method using the survival time of the adrenalectomized drake. The method had the advantage of being rapid, but, since the animals died in a matter of hours, the question arises as to the importance of the factor of stress. Thus, the test may measure the capacity of protecting animals against stress, as well as survival. The error of the potency ratio using relatively small numbers of animals (total of 20) appeared to be in the range of 33 per cent at  $P = 0.05$ . The method appears to be relatively sensitive, but has not been applied to crystalline material, so that it is difficult to get a measure of its relative sensitivity.

**POTASSIUM METABOLISM.** For studies on electrolyte metabolism, the potassium intoxication test has been suggested but not developed into an adequate bioassay due to the fact that the variation in sensitivity of adrenalectomized animals to potassium is great. In controlled experiments using adrenalectomized rats, it has been possible to detect about 0.75 mg. of desoxycorticosterone acetate per animal with a potassium intoxication test.<sup>6</sup> The accuracy of the assay using 20 animals on a standard and 20 on an unknown would probably not be any greater than  $\pm 50$  per cent, but not enough data is available to evaluate the data with certainty.

*Sodium Metabolism.* Although a number of methods have been described dealing with sodium excretion, no statistical evaluation of the methods are available. In a recent study,<sup>13</sup> normal dogs were employed and it was found that about 0.7 mg. of desoxycorticosterone acetate could be detected, but the error of estimation was not evaluated. Preliminary studies in the author's laboratory on rats, using radiosodium, have indicated a relatively sensitive and reproducible method, which is being studied at the present time.

*Carbohydrate Metabolism.* In the category of carbohydrate metab-

olism, there are methods dealing with the deposition of glycogen in the liver of the fasting adrenalectomized rat and mouse, the work test of Ingle, and the anti-insulin action of adrenal cortical material. With the exception of the last test, detailed statistical summaries are included. The anti-insulin test has been found to detect 0.5 mg. of corticosterone, 17-hydroxycorticosterone, and 17-hydroxy-11-dehydrocorticosterone.<sup>9</sup> The sensitivity is perhaps greater than 0.5 mg. per animal, but the variability has been found to be great in some unpublished work in the author's laboratory.

*Stress Tests.* Among the stress tests for adrenal cortical material, there are such tests as exposure to low environmental temperatures, histamine, typhoid toxin, and the effect of restraining animals. Of the various methods, only the cold test has been subjected to statistical analysis and these studies will be discussed in some detail.

### *Statistical Methods*

The various data discussed in this paper have been submitted to statistical analysis by known reliable methods. A brief description of these methods is in order.

Standard deviations have been calculated by the following formula:

$$S.D. = \sqrt{\frac{\sum(d)^2}{n-1}}$$

where *S.D.* = Standard Deviation

*d* = Individual Deviations from the Mean

*n* = number of observations.

Standard error of the mean has been calculated by the following formula:

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

where *S.E.* = Standard Error of the Mean.

The regression coefficients have been calculated by the method of Fischer,<sup>8</sup> according to the following formula:

$$Y = a + b(x - \bar{x})$$

where *b* = slope =  $\frac{\sum y(x - \bar{x})}{\sum (x - \bar{x})^2}$

*a* =  $\bar{y}$  = mean of *y*  
 $\bar{x}$  = mean of *x*.

The relative potencies have been determined by the method of Irwin<sup>12</sup> by the following formula, after proving that the slopes of the regression lines are not significantly different:

$$\log \text{ potency ratio} = \bar{x}_s - \bar{x}_u + \frac{\bar{y}_u - \bar{y}_s}{b_c}$$

where  $b_c = \frac{[\sum y(x - \bar{x})]_s + [\sum y(x - \bar{x})]_u}{[\sum (x - \bar{x})^2]_s + [\sum (x - \bar{x})^2]_u}$

$\bar{x}_s$  = mean of  $x$  of standard

$\bar{x}_u$  = mean of  $x$  of unknown

$\bar{y}_s$  = mean of  $y$  of standard

$\bar{y}_u$  = mean of  $y$  of unknown.

The error of the potency ratio was calculated by the following formula:

$$\lambda = \frac{\bar{S.D.}}{b_c} \sqrt{\frac{1}{m_s} + \frac{1}{m_u}}$$

where  $S.D.$  = weighted mean standard deviation.

The antilog of 2 times  $\lambda$  gives the high limit of error at  $P = 0.05$  above the value determined, while the antilog of the reciprocal gives the lower limit at  $P = 0.05$ .

The method of Bliss has been employed in a few instances and has been described in detail.<sup>2</sup> The experimental design consists of two concentrations each of the unknown and standard, so that the following proportion holds:  $\frac{U_1}{U_2} = \frac{S_1}{S_2}$ . From the individual observations, the slope, the relative potency, the error of the relative potency, and the deviation from parallelism of the two lines may be calculated. This latter step is essential, since the comparison is not valid unless the slopes of the lines are not significantly different.

#### *Analysis of a Growth-Survival Method in Adrenalectomized Rats*

For the analysis of this method, some data\* dealing with the survival and growth of rats adrenalectomized at 28 days of age were employed. The operated animals were treated for 20 days with either crystalline adrenal cortical steroids or adrenal cortical extracts. The results of the statistical evaluation are presented in TABLE 1 and summarize the relative potency of

TABLE 1  
THE RELATIVE POTENCY OF ADRENAL CORTICAL STEROIDS AND AN ADRENAL EXTRACT  
ON THE BASIS OF A GROWTH METHOD (20 DAY TEST)\*

Material	Number of animals	Slope	Relative potency	Error ( $P = 0.05$ ) %	" $p$ "
17-Hydroxy 11-dehydro-corticosterone	26	28.2	100	—	—
17-Hydroxy-corticosterone	18	43.2	219	—34, +51	0.365
Corticosterone	14	49.6	108	—32, +47	1.263
Lipo-Adrenal L-7234-35	29	39.5	—	—	0.788

\* From data kindly submitted by Dr. M. H. Kuizenga.

the three adrenal cortical steroids, 17-hydroxy-11-dehydrocorticosterone, 17-hydroxycorticosterone, and corticosterone. Calculations on an extract of the adrenal cortex is included to indicate the fact that the slope is essentially similar to that of 17-hydroxy-11-dehydrocorticosterone. In TABLE 2,

\* These data were kindly made available to me by Dr. Kuizenga of the Upjohn Company.

TABLE 2  
RELATIONSHIP BETWEEN SURVIVAL AND GROWTH OF THE ADRENALECTOMIZED RAT  
TREATED WITH ADRENAL CORTICAL STEROIDS AND EXTRACT\*

Material administered	Amount of material administered per rat per day	Mean weight gain in 20 days	Fraction of rats surviving 20 days
	$\mu\text{g.}$		
Lipo Extract	0.1 (W.U.)†	32.9	10/20
17-Hydroxy 11-dehydrocorticosterone	100 156	18.9 25.8	9/9 5/5
17-Hydroxy-corticosterone	100 200	27.8 41.6	9/13 10/10
Corticosterone	100 250	15.6 36.0	7/10 9/10

\* From data kindly submitted by Dr. M. H. Kuizenga.

† W. U. = Work Unit, an arbitrary unit.

we have an indication that there is not necessarily a direct relationship between the absolute weight gain of a treated adrenalectomized animal and its survival. Thus, at a time when some animals treated with Lipo-extract averaged gains of 32.9 grams in a 20 day period, only 10 of the 20 animals in the group survived 20 days, while similar animals treated with 17-hydroxy-11-dehydrocorticosterone showed gains of only 18.9 grams in 20 days, but 100 per cent survival. These illustrations are only to be taken as preliminary indications and the point should be submitted to further study.

#### *Analysis of Methods Dealing with Carbohydrate Metabolism*

*Use of the Adrenalectomized Rat.* The deposition of glycogen in the fasting adrenalectomized rat is the most precise method available for the bioassay of adrenal cortical material but not the most sensitive. Since the introduction of this method by Reinecke and Kendall,<sup>18</sup> it has been used widely. In this paper, the data of Pabst *et al.*<sup>16</sup> and Olson *et al.*<sup>14</sup> are considered in detail.

The dosage range employed by Pabst *et al.*<sup>16</sup> is illustrated in TABLE 3.

TABLE 3  
QUANTITIES OF ADRENAL CORTICAL STEROIDS USED FOR STUDIES ON RELATIVE POTENCIES\*

Compound	Amount of steroid per rat
	$\mu\text{g.}$
17-Hydroxy-11-dehydrocorticosterone	250 to 1000
Corticosterone	350 to 1000
17-Hydroxycorticosterone	125 to 1000
11-Dehydrocorticosterone	350 to 1000

\* Pabst *et al.*<sup>16</sup>

The minimum amount of material that could be used per animal is in the range of 250 micrograms equivalent of 17-hydroxy-11-dehydrocorticosterone.

TABLE 4 illustrates the utility of the method when relatively large num-

TABLE 4

THE RELATIVE ACTIVITIES OF VARIOUS ADRENAL CORTICAL STEROIDS EXPRESSED IN TERMS OF THE ACTIVITY OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE\*

Compound	Number of rats	$b \pm S.E.$	Potency ratio	Error ( $P = 0.05$ ) %	" $p$ "
17-Hydroxy-dehydrocorticosterone	50	$1.848 \pm 0.191$	100	—	—
Corticosterone	40	$1.677 \pm 0.183$	54	-13; +15	0.602
11-Dehydrocorticosterone	40	$2.229 \pm 0.251$	48	-10; +12	1.202
17-Hydroxycorticosterone	59	$1.963 \pm 0.131$	155	-12; +14	0.005

\* Calculated from data of Pabst *et al.*<sup>18</sup>

bers of animals are employed. For each compound, between 40 and 59 animals were employed at various concentrations of the material. In each instance the slope of the compound studied did not differ significantly from the slope of the reference compound, 17-hydroxy-11-dehydrocorticosterone. The relationship considered was that of the logarithm of the dose and the response. It is seen that both corticosterone and 11-dehydrocorticosterone are less active than 17-hydroxy-11-dehydrocorticosterone, but do not differ in activity among themselves. 17-Hydrocorticosterone is significantly more active than 17-hydroxy-11-dehydrocorticosterone. Of interest is the low order of error of the potency ratio. The maximum errors found was -13 to +15 per cent at  $P = 0.05$ .

The utility of the method is again illustrated in TABLE 5, where the potency

TABLE 5

THE ACTIVITY OF ADRENAL CORTICAL EXTRACTS IN TERMS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE (COMPOUND E)\*

Compound or extract	Number of animals	$b \pm S.E.$	Grams of tissue equivalent to 1 mg. Compound E	Error ( $P = 0.05$ )	" $p$ "
				%	
17-Hydroxy-11-dehydrocorticosterone	50	$1.848 \pm 0.191$	—	—	—
Hog	39	$2.244 \pm 0.187$	93.4	+14; -12	1.760
Beef	37	$2.134 \pm 0.467$	214.1	+15; -13	0.608

\* Calculated from data of Pabst *et al.*<sup>18</sup>

of hog and beef adrenal cortical extracts, on a weight basis, is presented in terms of 17-hydroxy-11-dehydrocorticosterone. A significantly greater activity was found for the hog extract and a low error in the potency ratio at  $P = 0.05$ , and no departure from parallelism of the respective slopes.

Of interest is the reproducibility of the method when smaller numbers of

animals are employed. In TABLE 6 such results are illustrated. Again, 17-hydroxy-11-dehydrocorticosterone was employed as the reference com-

TABLE 6  
THE RELATIVE POTENCY OF ADRENAL CORTICAL STEROIDS COMPARED TO 17-HYDROXY-11-DEHYDROCORTICOSTERONE\*

Compound	Slope	Total number or animals	Potency ratio	Error ( $P = 0.05$ )	" $q$ "
			%	%	
11-Hydroxycorticosterone	2.115	40	155	+29; -22	1.280
11-Dehydrocorticosterone	2.352	40	57.1	+25; -20	0.118
11-Hydroxycorticosterone	2.369	20	139	+34; -26	2.109
11-Hydroxycorticosterone	1.479	20	137	+96; -49	0.145
11-Dehydrocorticosterone	2.110	20	50	+48; -33	0.031
11-Dehydrocorticosterone	2.602	20	64.1	+30; -23	1.733

\* Calculated from data of Pabst *et al.*<sup>15</sup>

pound, and in two comparisons a total of 40 animals were employed, while in four tests a total of 20 animals were used. The Bliss design<sup>2</sup> was used in these instances.

In all comparisons, no departure from parallelism was detected. With a total of 40 animals at  $P = 0.05$ , an approximate error of  $\pm 24$  per cent can be expected, while with the use of 20 animals an approximate error of  $\pm 43$  per cent at  $P = 0.05$  is indicated. With a total of 80 animals, an error of approximately  $\pm 15$  per cent was found.

The data presented concerning the rat glycogen method thus far are those of Pabst *et al.*<sup>15</sup> It is of interest to compare these to the data of Olson *et al.*<sup>14</sup> The latter workers employed a similar method and their data were recalculated to conform with the calculations on the data of Pabst *et al.*<sup>15</sup> TABLE 7 illustrates the dosage range of the crystalline compounds used by Olson

TABLE 7  
AMOUNTS OF CRYSTALLINE ADRENAL CORTICAL STEROIDS EMPLOYED BY OLSEN *et al.*<sup>14</sup>

Compound	Amounts of steroid used
	$\mu\text{g.}$
Corticosterone	470 to 1240
11-Dehydrocorticosterone	390 to 1740
17-Hydroxy-11-dehydrocorticosterone	330 to 1270
17-Hydroxycorticosterone	440 to 1080

*et al.*<sup>14</sup> The calculations are presented in TABLE 8. The order of activity of the various compounds and the errors of the potency ratios agree rather well with those of Pabst *et al.*<sup>15</sup> However, the data on 17-hydroxycorticosterone could not be utilized, since the slope differed significantly from that of 17-hydroxy-11-dehydrocorticosterone.

*Use of the Adrenalectomized Mouse.* The rat glycogen method described has a relatively low order of error and has the further advantage that the



TABLE 8

THE RELATIVE ACTIVITIES OF ADRENAL CORTICAL COMPOUNDS EXPRESSED IN TERMS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE\*

Compound	Number of animals	<i>b</i>	Potency ratio	Error ( <i>P</i> = 0.05) = %
11-Dehydro-17-hydroxy-corticosterone	36	1.82	100	—
Corticosterone	23	1.88	75	—16 to +20
17-Hydroxycorticosterone	26	3.20	—	—
11-Dehydrocorticosterone	37	2.05	88	—13 to +16

\* Calculated from data of Olsen *et al.*<sup>14</sup>

crystalline compounds and the extracts studied have similar slopes when considered as a logarithm dose-response relationship. However, the amount of material needed is rather large. In order to detect smaller amounts of material, particularly such as is needed for the determination of cortin-like material in urine, it became necessary to devise a more sensitive method. The mouse glycogen method is such a method and has been studied by Eggleston *et al.*,<sup>6</sup> Dorfman *et al.*,<sup>3</sup> and Venning *et al.*<sup>19</sup>

From the data in TABLE 9, which presents the regression coefficients, and

TABLE 9

THE LINEAR REGRESSION FORMULAE OF 11-DEHYDROCORTICOSTERONE, 17-HYDROXY-11-DEHYDROCORTICOSTERONE, URINARY EXTRACT, AND PIG ADRENAL CORTICAL EXTRACT USING THE RELATIONSHIP BETWEEN LOGARITHM OF THE DOSE AND THE RESPONSE\*

Material administered	Linear regression formulae	Remarks
11-Dehydrocorticosterone	$Y = 6.38x - 8.15^1$ $Y = 3.54 \pm 1.09 + 6.46 \pm 0.81 (x - \bar{x})$	
17-Hydroxy-11-dehydrocorticosterone	$Y = 22.23x - 27.36$ $Y = 7.05 \pm 0.74 + 22.23 \pm 3.08 (x - \bar{x})$	From 3 points, 20, 40, and 80 micrograms
	$Y = 6.66x - 4.81$	From 4 points, 5, 10, 20, and 40 micrograms
Urinary extract	$Y = 4.88x - 7.80$ $Y = 1.96 \pm 1.09 + 4.88 \pm 0.72 (x - \bar{x})$	
Pig adrenal cortical	$Y = 4.79x + 2.38$	From 4 points, 0.01, 0.02, 0.04, and 0.08 cc. $x = \log 100 \times \text{dose}$

\* From data of Dorfman *et al.*<sup>3</sup>

FIGURE 2, which is the graphic representation of the logarithm of the dose-response relationship, it is seen that the slopes of such diverse materials as 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, a hog adrenal cortical extract, and a urinary extract do not differ significantly.

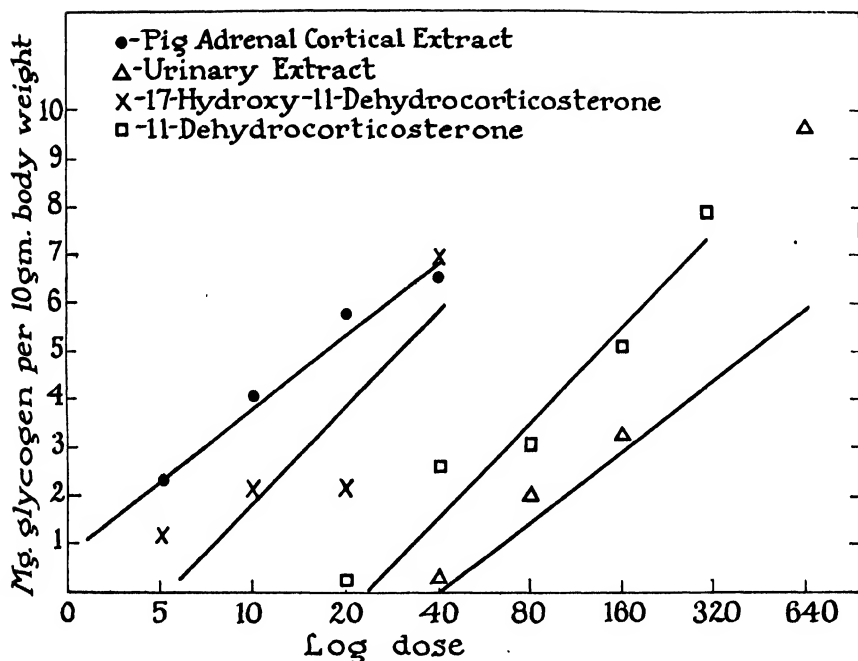


FIGURE 2. Liver glycogen responses to increasing doses of pig adrenal cortical extract, urinary extract, 17-hydroxy-11-dehydrocorticosterone, and 11-dehydrocorticosterone. Doses of pig adrenal cortical extract are plotted as the logarithm of 500 times the dose in cc. of oil solution. The urinary extract is plotted as the logarithm of the dose in cc. and the doses of the two adrenal cortical steroids are represented as the logarithm of the dose in micrograms.

Studies were conducted as to the error one may expect if approximately 5 or 10 animals are used on an unknown preparation and the values referred to a standard, in this case, 11-dehydrocorticosterone. This is of interest, since in normal human urine only enough material is available for the use of 5 to 10 animals when a 48-hour sample is used. The predictable error at  $P = 0.05$  is  $-72$  to  $+260$  for 5 animals. In TABLE 10, it is seen that in 11 of 12 experiments (from 3 to 6 animals) the error ranged from 41 per cent low to 230 per cent high, and in one instance the value was 431 per cent high. In TABLE 11 a similar sort of analysis is presented for approximately 10 animals.<sup>8-14</sup> Here, the errors ranging from  $-50$  to  $+150$  were found in 16 experiments. The predictable error is actually  $-59$  to  $+150$  per cent when  $P = 0.05$ .

It was seen that a linear relation is apparent when the logarithm of the dose-response is plotted up to values of approximately 10 mg. of glycogen per 10 g. of body weight. However, if responses are considered up to approximately 20 mg. of glycogen per 10 g. of body weight for 17-hydroxy-11-dehydrocorticosterone and a pig adrenal cortical extract, the curve assumes an exponential character when plotted as logarithm of the dose *versus* response. If the data are reconsidered, plotted as the relationship between the logarithm of the dose and the logarithm of the response, a linear relationship is found. This is illustrated in FIGURE 3. Using this new relationship,

TABLE 10

VARIATIONS IN RESPONSE TO 80 MICROGRAMS OF 11-DEHYDROCORTICOSTERONE\*

(Values read from standard 11-dehydrocorticosterone curve. Approximately 5 mice per group.)

Number of mice	Amount found: micrograms	Errors
		%
3	113	+41
4	49	-39
4	103	+29
4	105	+31
5	425	+431
5	100	+25
5	63	-21
5	265	+230
6	65	-19
6	130	+63
6	70	-13
6	70	-13

\* From data of Dorfman *et al.*<sup>3</sup>

TABLE 11

VARIATIONS IN RESPONSE TO 80 MICROGRAMS OF 11-DEHYDROCORTICOSTERONE\*

(Values read from standard 11-dehydrocorticosterone curve. Approximately 10 mice per group.)

Number of mice	Amount found: micrograms	Errors
		%
8	200	+150
8	40	-50
8	49	-39
10	44	-45
10	78	-3
10	130	+63
10	170	+113
10	130	+63
10	163	+138
10	160	+100
10	81	+1
11	118	+48
11	123	+54
11	83	+38
12	49	-39
14	60	-25

\* From data of Dorfman *et al.*<sup>3</sup>

no significant difference was found in the slopes of the hog adrenal cortical extract and 17-hydroxy-11-dehydrocorticosterone up to a response of approximately 20 mg. of glycogen per 10 g. of body weight.

The relative activity of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone was studied by Dorfman *et al.*<sup>4</sup> and by Venning *et al.*<sup>10</sup> These comparisons are tabulated in TABLE 12. The data of Venning *et al.*<sup>10</sup> have been recalculated by the statistical methods employed in the paper of Dorfman *et al.*<sup>4</sup> The method employed by Dorfman *et al.*<sup>4</sup> and originally suggested by Eggleston *et al.*<sup>6</sup> consisted in protecting the fall

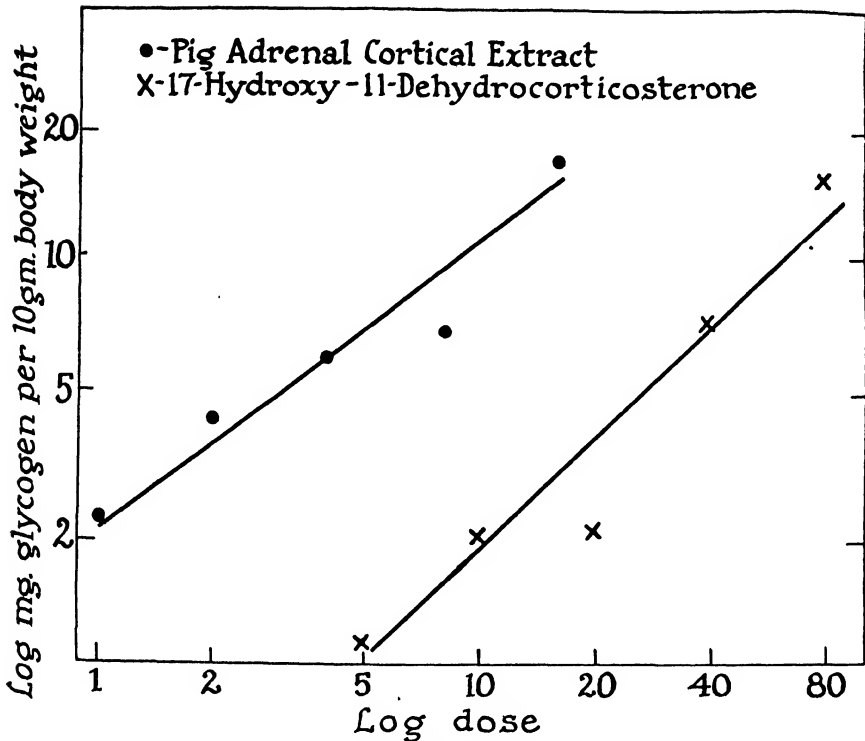


FIGURE 3. Liver glycogen responses to increasing doses of pig adrenal cortical extract and 17-hydroxy-11-dehydrocorticosterone. Doses of pig adrenal cortical extract are plotted as the logarithm of 100 times the dose in cc. of oil solution while doses of 17-hydroxy-11-dehydrocorticosterone are expressed as the logarithm of the weight in micrograms. The formula for 17-hydroxy-11-dehydrocorticosterone was  $\bar{Y} = 1.317 x - 0.961$  and the formula for the pig adrenal cortical extract was  $\bar{Y} = 0.659 x + 0.370$ .

TABLE 12  
COMPARISON OF THE ACTIVITIES OF 11-DEHYDROCORTICOSTERONE (A) AND 17-HYDROXY-11-DEHYDROCORTICOSTERONE (E) BY THE MOUSE GLYCOGEN METHOD

Method	Total number of animals	Potency of A in % of E	Error ( $P = 0.05$ )
Protect fall of liver glycogen (Dorfman <i>et al.</i> ) <sup>4</sup>	238	26	% -25; +33
Build up liver glycogen (Venning <i>et al.</i> ) <sup>10</sup>	73	29	-17; +20

in liver glycogen in a fasting adrenalectomized mouse, while the method of Venning *et al.*<sup>10</sup> consisted in building up the glycogen of the liver. Two points are of interest. First, it is seen that the relative potency of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone by the two methods are in good agreement. Thus, 11-dehydrocorticosterone was 26 and 29 per cent as active as 17-hydroxy-11-dehydrocorticosterone by the methods of Dorfman *et al.*<sup>4</sup> and Venning *et al.*,<sup>10</sup> respectively. Secondly, the

method of Venning *et al.*<sup>19</sup> appears to have the higher accuracy as seen by the fact that a lower error range of the potency ratio was found with fewer animals.

The sensitivity of the mouse glycogen method is in the range of 10 micrograms of 17-hydroxy-11-dehydrocorticosterone, which means the method is about 25 times as sensitive as the rat glycogen method.

*The Ingle Work Test.* When the Ingle work test is employed with the adrenalectomized-nephrectomized rat, the method appears to measure carbohydrate effects. When 15 animals are used on an unknown preparation and the values referred to 135 animals on the reference compound, accuracy is in the order of  $\pm 20$ .<sup>15</sup> The sensitivity of the method is in the range of 400 micrograms of 17-hydroxy-11-dehydrocorticosterone (TABLE 13).

TABLE 13

THE RELATIVE POTENCIES OF ADRENAL CORTICAL STEROIDS IN TERMS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE ON THE BASIS OF THE WORK TEST\*

Compound	Potency ratio 17-Hydroxy-11- dehydro- corticosterone taken as 100	Error ( $P =$ 0.05)
		%
17-Hydroxycorticosterone	192	$\pm 20$
Corticosterone	63	
11-Dehydrocorticosterone	48	
11-Desoxycorticosterone	2	
17-Hydroxy-11-desoxycorticosterone	2	

\* From data of Pabst *et al.*<sup>15</sup>

*The Cold Test—An Example of a Stress Test.* Beginning with the work of Hartman *et al.*,<sup>10</sup> the increased sensitivity of adrenalectomized animals to low environmental temperatures has been used as a means of determining adrenal cortical activity. A detailed description of the method has been published using operated mice and rats.<sup>5</sup> Since the rat was found to be the most reliable, our discussion will be confined to studies on the rat.

The test can be characterized as simple, rapid, and sensitive. Its usefulness is marred by an erratic variation in sensitivity of animals from group to group.

The use of the cold test can be illustrated by the assay of urinary extracts in terms of a crystalline standard, specifically 11-dehydrocorticosterone, and studies on the relative activities of various adrenal cortical steroids.

Some of the studies were done when the unknown and standard were run simultaneously, and in others, comparisons were made from group to group, only if two conditions were fulfilled in the various runs:

- A. A minimum standard dose of 11-dehydrocorticosterone gave a statistically significant response.
- B. The mean survival of the control animals did not differ significantly among the groups used.

Such rigid control was necessary, since in about one third of the runs the response was extremely low or zero. Although small amounts of urinary extract gave good responses in the cold test, it was found frequently that, after the maximum increase in survival time was attained, further increases in dosage caused a decreased response. Thus, in dealing with urinary extracts, it is necessary to be sure that one is working in the region of the ascending portion of the dose-response curve.

FIGURES 4, 5, and 6 illustrate the responses of 17-hydroxy-11-dehydro-

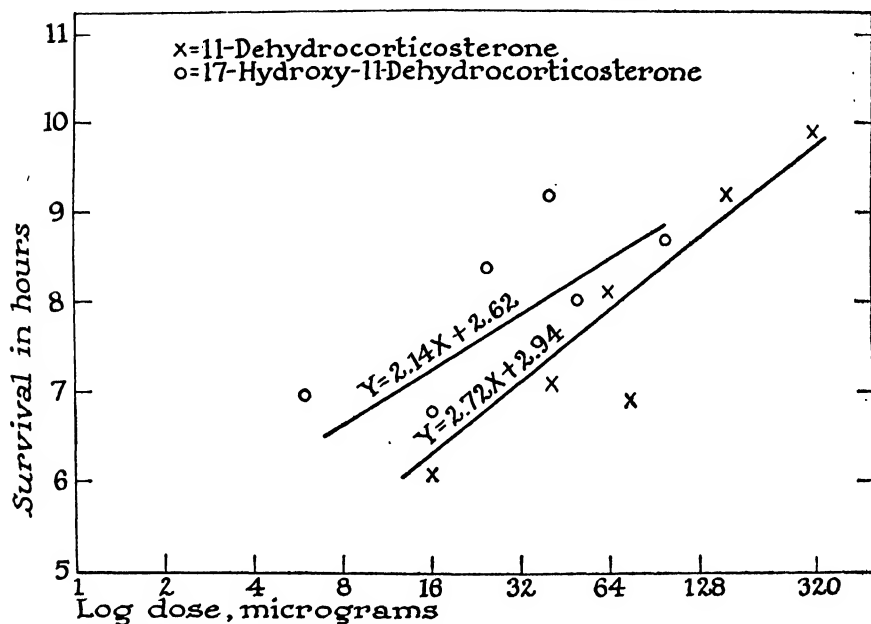


FIGURE 4. Log dose-response curves for 11-dehydrocorticosterone (162 rats) and 17-hydroxy-11-dehydrocorticosterone (113 rats).

Control mean survival was 5.7 hours.

corticosterone, corticosterone, and 11-desoxycorticosterone acetate, respectively, as compared to 11-dehydrocorticosterone. No significant differences were found in the respective slopes.

In FIGURES 7 and 8 the comparative responses of an urinary extract to 11-dehydrocorticosterone are illustrated. The relative potencies are tabulated in TABLE 14. A good agreement in the three assays was found, as seen by the fact that, in three assays, 600, 660, and 650 cc. respectively, of urinary extract, were found to be equal in activity to that of one milligram of 11-dehydrocorticosterone.

The determinations of the relative activities of various adrenal cortical steroids are presented in TABLE 15. The expected error of the potency ratio is higher than that for the glycogen methods. 11-Dehydrocorticosterone is about one-third as active as 17-hydroxy-11-dehydrocorticosterone, while corticosterone and 11-desoxycorticosterone acetate are one-twelfth as active by this method.

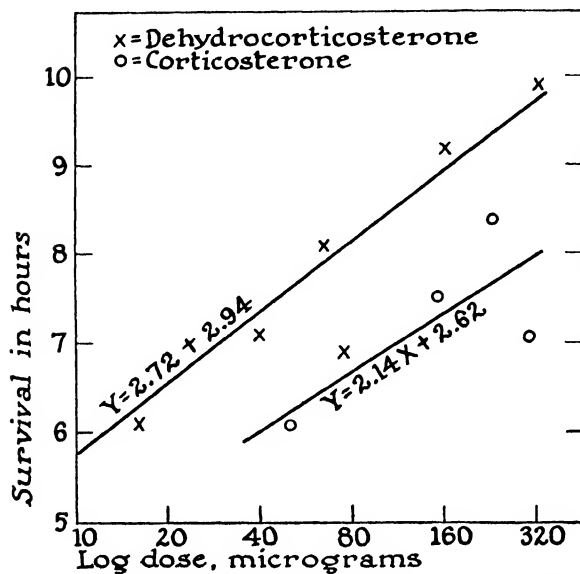


FIGURE 5. Log dose-response curves for 11-dehydrocorticosterone (162 rats) and corticosterone (76 rats). Control mean survival was 5.7 hours.

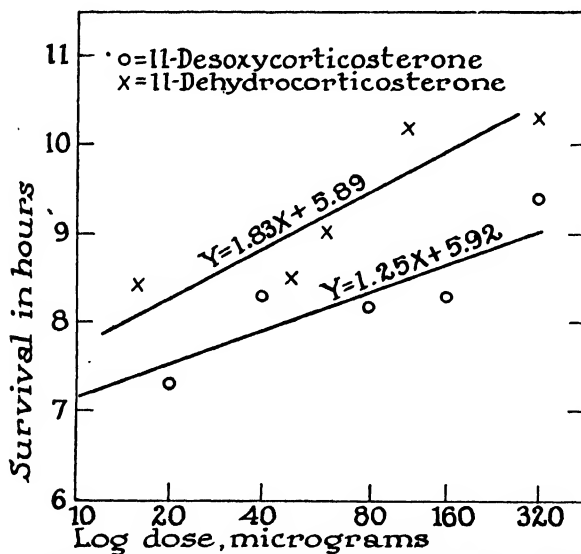


FIGURE 6. Log dose-response curves for 11-dehydrocorticosterone (83 rats) and 11-desoxycorticosterone acetate (52 rats). Control mean survival was 7.3 hours.

#### *Comparative Activity of Compounds of Various Tests*

A summary of the comparative activities of the various adrenal cortical steroids is given in TABLE 16. The activity of 17-hydroxy-11-dehydro-

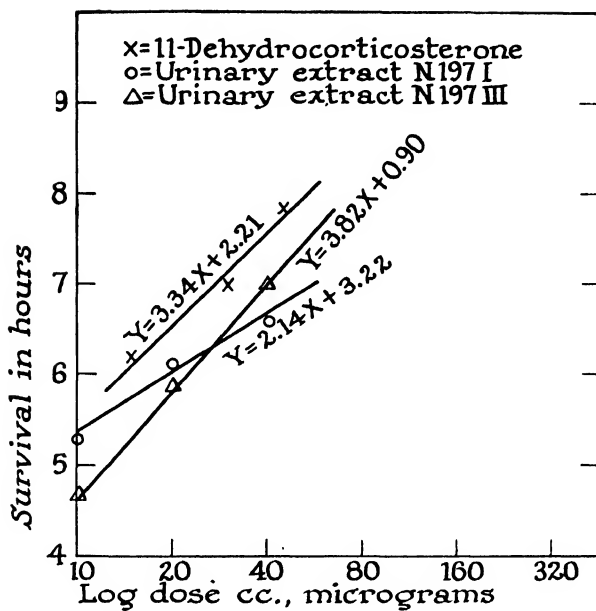


FIGURE 7. The total number of animals used for the curve of 11-dehydrocorticosterone was 51. Each cross represents from 15 to 20 animals. The regression lines were determined by the method of least squares.

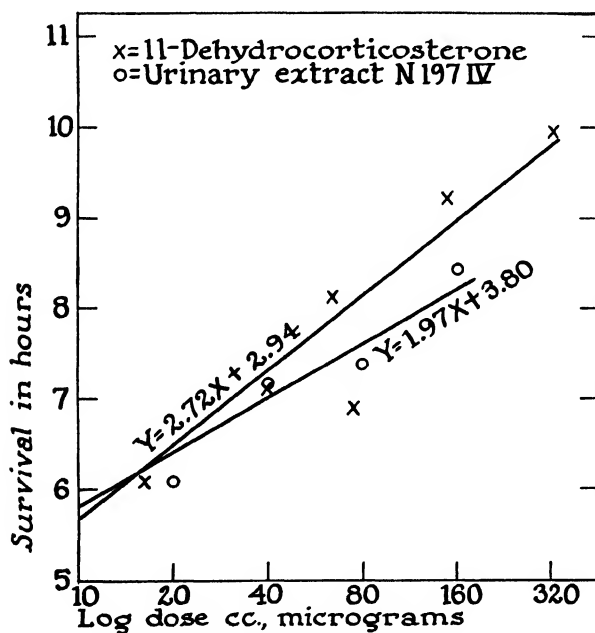


FIGURE 8. The total number of animals used to determine the standard curve was 162. Each cross represents from 7 to 97 animals.



TABLE 14  
ASSAY OF POOLED URINARY EXTRACT IN TERMS OF 11-DEHYDROCORTICOSTERONE\*

Date	Experiment number	Mg. of 11-Dehydrocorticosterone per liter	Error range ( $P = 0.05$ )	
			Per cent low	Per cent high
9-14-44	I	0.60	50	102
9-28-44	III	0.66	40	67
1-24-45	IV	0.65	26	35

\* From data of Dorfman *et al.*<sup>5</sup>

TABLE 15  
THE RELATIVE POTENCIES OF VARIOUS ADRENAL CORTICAL STEROIDS ON THE BASIS OF A COLD PROTECTION TEST\*

Compound	Total number of rats	Amount of compound equivalent to one microgram of 11-Dehydrocorticosterone	Error of estimate $P = 0.05$		Remarks
			Low	High	
			%	%	
17-Hydroxy-11-dehydrocorticosterone	275	0.5	32	46	Compounds run simultaneously
	159	0.3	49	93	
	76	0.2	48	92	
	76	0.3	48	92	
Corticosterone	238	3.8	35	55	
11-Desoxy-corticosterone acetate	192	4.0	42	71	Compounds run simultaneously
	135	4.7	54	115	

\* From data of Dorfman *et al.*<sup>4</sup>

TABLE 16  
COMPARATIVE ACTIVITIES OF VARIOUS ADRENAL CORTICAL STEROIDS BY VARIOUS METHODS EXPRESSED IN RELATION TO THAT OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE

Compound	Relative potency in terms of 17-Hydroxy-11-dehydrocorticosterone				
	Glycogen rat	Glycogen mouse	Ingle work test	Cold protection test	Growth
17-Hydroxy-11-dehydrocorticosterone	100	100	100	100	100
17-Hydroxycorticosterone	155	—	192	—	219
11-Dehydrocorticosterone	47	28	48	33	—
Corticosterone	54	—	63	9	108
11-Desoxycorticosterone	—	2	2	8	—
17-Hydroxy-11-desoxycorticosterone	—	—	2	—	—

corticosterone has been set at 100, and the values for the other compounds are expressed as percentages of the activity of this hormone. Only those methods are listed where suitable data are available for the estimation.

*Comparative Sensitivity and Reproducibility of Various Methods.* A summary of the comparative sensitivity and reproducibility of the various methods is presented in TABLE 17. The methods for the assay of materials

TABLE 17  
THE COMPARATIVE SENSITIVITY AND REPRODUCIBILITY OF VARIOUS METHODS FOR THE ASSAY OF ADRENAL CORTICAL MATERIAL

Test	Animal used	Minimum amount of steroid needed per animal		Approximate number of animals on standard	Approximate number of animals on unknown	Error ( $P = 0.05$ )
		"E"*	DCA†			
		μg.	μg. 700			%
Sodium excretion	Normal dog	—	—	—	—	—
Potassium intoxication	Adrenal X rat	—	750	20	20	±100
Insulin sensitivity	Normal mouse	500	—	—	—	—
Growth (20 days)	Adrenal X rat	2000	—	26 26	14 29	-32 to +51 -28 to +40
Glycogen	Adrenal X rat	250	—	40 20 10	40 20 10	±15 ±24 ±43
Glycogen	Adrenal X mouse	10	—	40 40	40 10	±20 +50 to +150
Work test	Adrenal X rat	400	—	135	15	±20
Cold test	Adrenal X rat	4	—	100 40	100 40	-35 to +65 -40 to +90

\* 17-Hydroxy-11-dehydrocorticosterone.

† 11-Desoxycorticosterone acetate.

on the basis of either sodium or potassium metabolism are not well enough established to define the expected errors. In the case of the potassium intoxication test, a rough approximation of ±100 per cent has been listed when 20 animals are used on both the standard and unknown. The amounts needed for this test, as well as sodium excretion in the normal dog, appears to be of a rather high order, 700 micrograms of desoxycorticosterone acetate.

The glycogen methods appear to be well established, with the rat method being less variable than the mouse methods, but also less sensitive. The work test, though not particularly sensitive, has a high precision. The cold

protecting test is extremely sensitive, but variable enough to decrease its value considerably.

### Summary

A survey was made of the methods that have been suggested for the bioassay of adrenal cortical activity from the standpoint of sensitivity and reproducibility. Methods which deal with electrolyte metabolism (sodium and potassium) have not been developed to a high degree of either accuracy or sensitivity.

Methods pertaining to carbohydrate metabolism have been studied in detail and submitted to statistical evaluation. The method of deposition of glycogen in the liver of the fasting adrenalectomized rat is characterized by a high precision, but a relatively low sensitivity. A minimum of 250 micrograms of 17-hydroxy-11-dehydrocorticosterone or its equivalent per animal is required in this test. When a total of 80 animals is employed, half on a standard and half on an unknown, an accuracy of  $\pm 15$  ( $P = 0.05$ ) can be obtained. The use of a total of 40 animals results in an accuracy of about  $\pm 24$  ( $P = 0.05$ ), while a total of 20 animals gives an accuracy of about  $\pm 43$  per cent ( $P = 0.05$ ). The use of the adrenalectomized mouse in place of the rat results in a 25-fold increase in sensitivity, but a decrease in precision.

The work test of Ingle using the nephrectomized-adrenalectomized rat, which is primarily dependent upon the influence on carbohydrate metabolism, has a sensitivity and reproducibility of the order of the rat glycogen method.

The cold protection test, an example of a stress test, is the most sensitive test, but has a lower order of accuracy; and its utility is decreased by the variability in sensitivity from group to group. The growth-survival method of bioassay needs further study for precise evaluation.

The comparative activities of adrenal cortical steroids as determined by the rat glycogen, the mouse glycogen, the work test, the cold test, and a growth method are presented.

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# SOME STUDIES ON THE ROLE OF THE ADRENAL CORTEX IN ORGANIC METABOLISM

By DWIGHT J. INGLE

*Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

This paper is concerned with the recent studies in our laboratories on, first, the metabolic effects of adrenal cortical extracts, pure steroids, and pure adrenocorticotrophic hormone, and second, the possible relationship of these effects to some of the metabolic changes which occur during stress.

## *Background*

My interest in this general problem was stimulated by the classical studies of Long and Lukens,<sup>1</sup> who found that pancreatic diabetes in laboratory animals could be alleviated by removal of the adrenal cortices. Large amounts of adrenal cortical extract caused an intensification of the diabetes either in the adrenalectomized-depancreatized animal<sup>2, 3</sup> or in the partially depancreatized, mildly diabetic rat.<sup>3</sup> When pure adrenal steroids were tested for their effects upon carbohydrate metabolism, it was found that 11-desoxycorticosterone was very weak in its diabetogenic and glycogenic activity and that the C-11-oxygenated steroids were much more potent.<sup>4, 5</sup> These studies were further extended by the demonstration of adrenal steroid diabetes in the normal force-fed rat.<sup>6</sup> In rats, the glucosuria always disappears when injections of the steroid are stopped.

The administration of large amounts of cortical extracts or of the C-11-oxygenated adrenal steroids causes an increase in the excretion of non-protein nitrogen, together with an increase in liver glycogen.<sup>4</sup> This is evidence for an increase in gluconeogenesis. It was first believed that the effects of adrenal cortical insufficiency and excess could be completely accounted for in terms of the amount of gluconeogenesis from protein, but further studies showed that the problem was more complex.<sup>7, 8</sup> In studies on the partially depancreatized rat given a constant food intake by stomach tube, Ingle and Thorn<sup>5</sup> found that a mild glycosuria could be abolished by adrenalectomy without any decrease in the amount of urinary nitrogen. Furthermore, the administration of 17-hydroxy-11-dehydrocorticosterone to such rats caused the excretion of much larger amounts of glucose than could be accounted for on the basis of the increase in urinary nitrogen, even if a carbon-to-carbon conversion of protein to carbohydrate was assumed. Such observations led to the conclusion that either the adrenal cortical hormone affects the conversion of fat to carbohydrate or it inhibits the utilization (oxidation, storage or conversion) of carbohydrate. Many investigators are unwilling to accept the assumption that a conversion of fat to carbohydrate occurs to any significant extent. It seems more reasonable to assume that the withdrawal of the cortical hormones in the diabetic animal permits some increase in the utilization of carbohydrate, and that an excess of the cortical hormones causes inhibition of utilization.

To summarize, the amounts of carbohydrate in the blood and tissues of

experimental animals are usually depleted in cortical hormone insufficiency and are increased above normal in cortical hormone excess. This correlation is based in part upon the effect of the C-11-oxygenated cortical steroids upon gluconeogenesis from non-carbohydrate sources and perhaps to a greater extent upon the utilization of carbohydrate. In addition, the adrenal cortical hormones may either directly or indirectly influence food intake, absorption, transport, and storage of carbohydrate. This evidence and a more detailed review of the earlier studies on the role of the cortical hormones in organic metabolism have been presented elsewhere.<sup>8</sup>

### *Current Problems*

#### *Some Metabolic Effects of Adrenal Steroids and ACTH*

*Adrenal Steroid Diabetes in the Normal Rat.* How are the effects of the cortical hormones upon the formation and the utilization of carbohydrate related? Drury<sup>9</sup> and Ingle<sup>10</sup> have each suggested that increased gluconeogenesis from protein might represent a compensatory response to a primary inhibitory effect of the cortical hormones on the utilization of carbohydrate. In pancreatic and phlorhizin diabetes and during starvation, there is an increase in protein catabolism which coexists with a decrease in the utilization of carbohydrate. In animals having pancreatic diabetes, control of the glycosuria by treatment with insulin restores a positive nitrogen balance. We<sup>11</sup> have compared the effects of insulin upon nitrogen balance in rats having adrenal steroid diabetes and upon similar animals having pancreatic diabetes. An unanticipated finding was the remarkable insulin resistance of those animals which had adrenal steroid diabetes. This is illustrated in FIGURE 1 by a normal rat which was force-fed a high carbohydrate diet (15 grams of available carbohydrate per day) and made severely diabetic by the administration of 5 mg. daily of 17-hydroxy-11-dehydrocorticosterone. The injection of up to 100 units of protamine zinc insulin per day failed to abolish the glycosuria. In a second animal made diabetic by the administration of 5 mg. daily of 17-hydroxycorticosterone, the administration of up to 1000 units daily of regular insulin failed to control completely the glycosuria. FIGURE 2 gives data on a similar animal in which the hyperglycemia and glycosuria were controlled by protamine zinc insulin without an effect upon the elevated level of urinary non-protein nitrogen. FIGURE 3 shows a typical response of a rat having severe pancreatic diabetes. Treatment with insulin effectively controlled the glycosuria and hyperglycemia and restored a positive nitrogen balance. Although the results of this experiment fail to support the hypothesis under test, they do not fully answer the question. The insulin resistance of the animals having adrenal steroid diabetes prevented our fully controlling the diabetes in all instances.

Which of the adrenal steroids are diabetogenic? In this laboratory we have induced adrenal steroid diabetes in normal rats with corticosterone,<sup>12</sup> 17-hydroxy-corticosterone,<sup>11, 12</sup> and 17-hydroxy-11-dehydrocorticosterone.<sup>11</sup> Massive doses of 11-desoxycorticosterone are diabetogenic in the partially depancreatized rat.<sup>5</sup> The compound 11-dehydrocorticosterone and the "amorphous fraction" of adrenal extracts have not been fully studied.

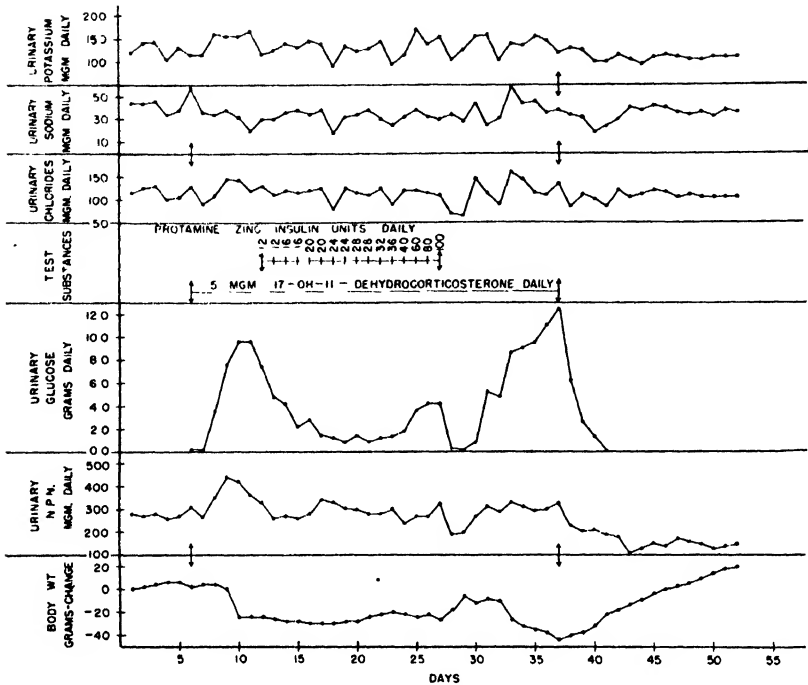


FIGURE 1. Adrenal steroid diabetes in a normal male rat.

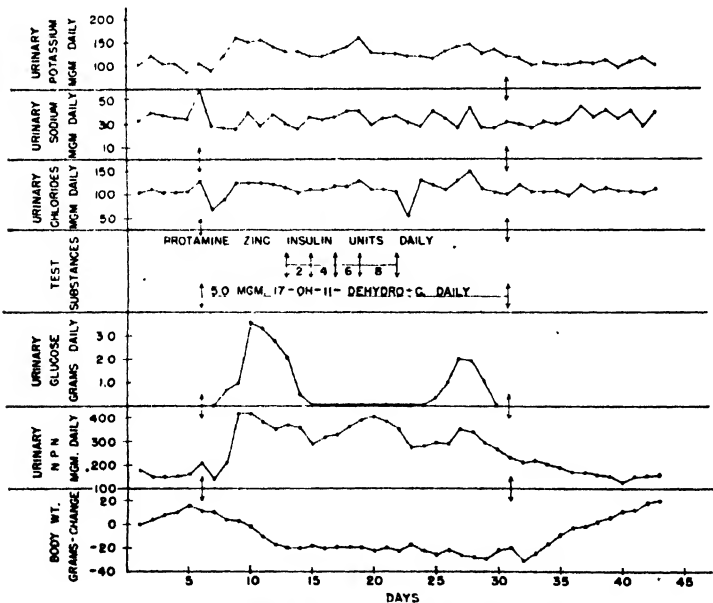


FIGURE 2. Adrenal steroid diabetes in a normal male rat.

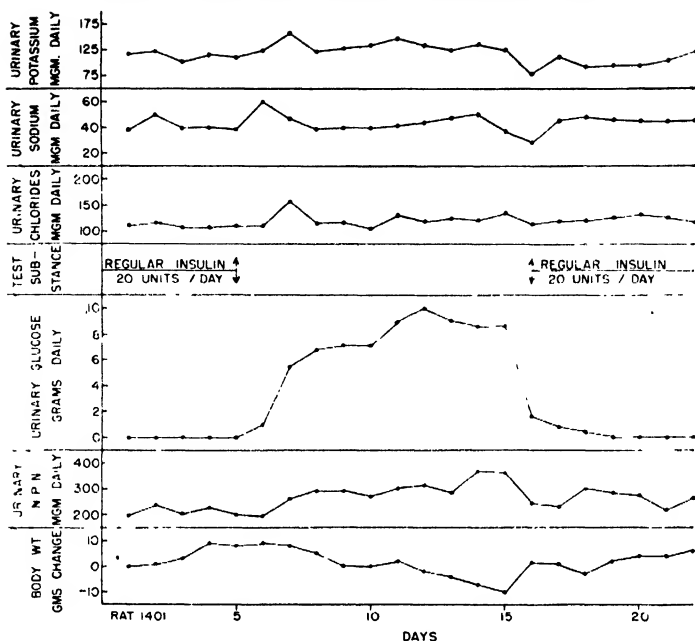


FIGURE 3. Pancreatic diabetes in a male rat.

*The Production of Hyperadrenocorticism by Adrenocorticotrophic Hormone in the Rat.* Is adrenal steroid diabetes a pharmacologic phenomenon only, or is it within the capacities of the animals' own adrenal cortices to secrete amounts of hormone which will cause similar effects? We have had the privilege of collaborating with Doctors Li and Evans, of the University of California, in studies on the biologic properties of pure adrenocorticotrophic hormone (ACTH). We have reported<sup>13</sup> the production of glycosuria and hyperglycemia in normal rats given ACTH. There was an increase in the excretion of nitrogen and potassium, inhibition of growth, and a manifestation of hyperglycemia and glycosuria just as occurred in similar animals having adrenal steroid diabetes. The results are illustrated in FIGURE 4. It is apparent that the adrenal cortices of rats can secrete the activity equivalent of very large amounts of adrenal cortical extracts or of pure adrenal cortical compounds. Additional evidence on the secretory capacity of the adrenal cortices has been reviewed elsewhere.<sup>14</sup>

The extent to which ACTH will alter the nitrogen balance of the experimental animal may be related to the nature of the diet. Ingle, Prestrud, Li, and Evans (unpublished) compared the effects of ACTH in normal animals force-fed high carbohydrate, high fat, and high protein diets in isocaloric amounts. There was no tendency for ACTH to cause a greater change in nitrogen balance in the rats which received the high protein diet. On the contrary, the administration of ACTH had its smallest effect upon nitrogen balance and body weight in rats force-fed the high protein diet, and its greatest effect upon similar animals maintained on the high fat diet. The



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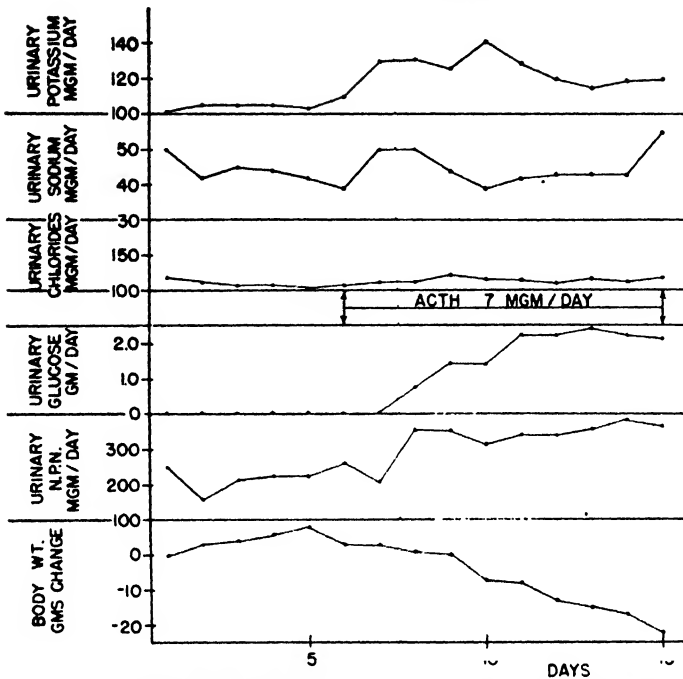


FIGURE 4. Glycosuria caused by adrenocorticotrophic hormone in a normal male rat.

changes in weight paralleled the results on nitrogen balance. The data are summarized in TABLES 1 and 2. Only a small number of animals were tested,

TABLE 1

THE RELATIONSHIP OF DIET TO THE EFFECT OF ACTH UPON URINARY NON-PROTEIN NITROGEN IN THE NORMAL RAT

Daily dose ACTH	Days treatment	*Average daily increase in urinary N.P.N. above control values					
		Increase above values of pre-injection period			Increase above values of control rats		
		High COH	High fat	High protein	High COH	High fat	High protein
mg.							
1	21	54	67	26	44	87	17
3	21	61	77	30	53	88	18
8	10	78	95	64	71	85	42

\* Two control and two experimental animals were represented at each level of dosage for each diet, except that 4 rats were given 8 mg. of ACTH per day on the high carbohydrate diet.

but the differences in response were striking and consistent, and are probably valid, thereby giving a basis for adding again to the growing list of interesting questions which relate to the effects of ACTH and the adrenal cortical hormones.

TABLE 2  
THE RELATIONSHIP OF DIET TO THE EFFECT OF ACTH UPON CHANGE IN WEIGHT IN THE NORMAL RAT. GRAMS CHANGE

Daily dose ACTH  mg.	Days treatment	*Average total change in weight during the injection period		
		High COH	High fat	High protein
1	21	-10	-17	24
3	21	-28	-33	-8
8	10	-25	-25	-17

\* Two control and two experimental animals were represented at each level of dosage for each diet, except that 4 rats were given 8 mg. of ACTH per day on the high carbohydrate diet.

Only the animals on the high carbohydrate diet developed glycosuria during the administration of ACTH in these experiments.

*The Effect of Adrenal Cortex Extract Upon the Glucose Tolerance of the Eviscerated Rat.* It is frequently assumed that the liver is the sole site of action of the cortical hormones upon carbohydrate metabolism. Is the "diabetogenic" effect of cortical extract mediated by the liver? A number of years ago,<sup>15</sup> cortical extract was found to have a definite effect upon the ability of the eviscerated-nephrectomized-adrenalectomized rat to work when treated with glucose. Russell<sup>16</sup> has shown that the utilization of glucose is increased in the adrenalectomized, functionally eviscerated rat. We have now shown, as is illustrated in FIGURE 5, that the intravenous administration of adrenal cortical extract to the eviscerated rat over a period of 24 hours decreases its tolerance for glucose given by constant infusion,

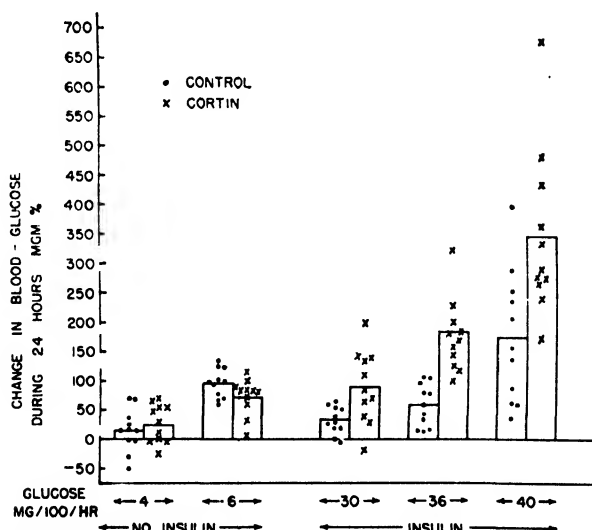


FIGURE 5. The effect of adrenal cortex extract upon the tolerance for intravenously administered glucose in the eviscerated rat. Averages and individual values.

We have observed this effect only in animals which were treated with insulin. The glucose utilization of similar animals without insulin has not been significantly changed by adrenal cortex extract. Is this effect identical with the "diabetogenic" action of the cortical hormones in intact animals? We do not know, but at present there is no reason to assume that it is different. It is a demonstration of an extrahepatic effect, pharmacologic or not, of adrenal cortical extract. How does this effect relate to the action of the cortical hormones in supporting the ability of the eviscerated-adrenalectomized rat to work? It should be interesting to study the effect of the cortical hormones upon glucose tolerance in the eviscerated, working animal.

*Adaptation to Adrenal Steroid and ACTH Administration.* The early effects of treatment with hormones may not be sustained throughout prolonged administration. This point has been neglected many times. It is an important principle in endocrinology that an appraisal of the physiologic properties of a hormone should be based upon an exploration of the full range of dose-response relationship and of time-response relationship. The limited availability of compounds, such as the adrenal steroids and ACTH, has restricted many studies to 24 or 48 hours and even less. It usually requires a latent period of 72 hours or longer for either the adrenal steroids or ACTH to cause glycosuria. The normal animal is capable of adaptation to the continued administration of a diabetogenic dose of either hormone, so that the glycosuria may disappear. It is not known whether the diabetic state could be sustained indefinitely by a larger dose. The administration of a dose of an adrenal steroid just large enough to induce a negative nitrogen balance in the normal force-fed rat does not sustain the negative nitrogen balance, as is illustrated in FIGURES 6 and 7. The negative nitrogen balance

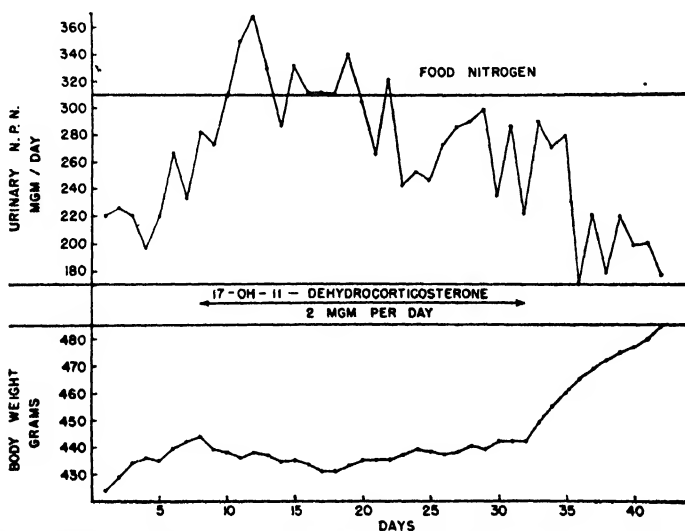


FIGURE 6. Adaptation to the effect of an adrenal steroid upon nitrogen balance in a normal force-fed male rat.

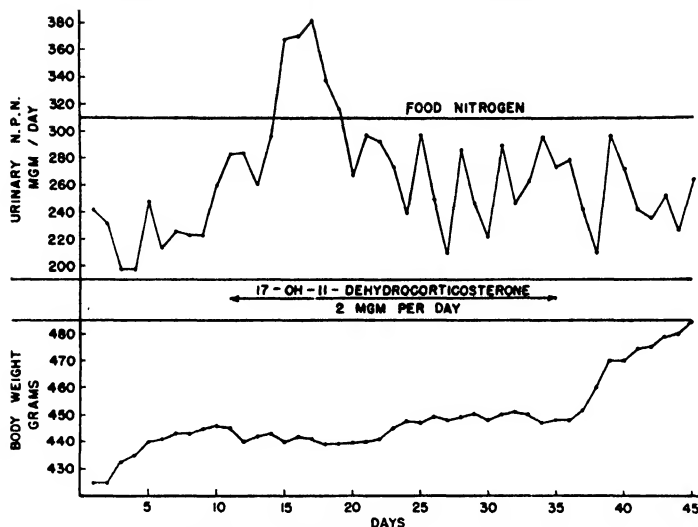


FIGURE 7. Adaptation to the effect of an adrenal steroid upon nitrogen balance in a normal force-fed male rat.

can be re-established by increasing the dose of the hormone. Almost all of the information on the glycogenic effect of adrenal hormones and ACTH has been derived from acute experiments. Can liver glycogen be sustained at a high level by the continued administration of the cortical hormones? Is liver glycogen high in animals having adrenal steroid diabetes, or does it fall to the abnormally low levels which characterize pancreatic diabetes? These and similar questions will require studies of longer duration.

The animal normally lives with its own hormones quietly carrying out their functions with homeostatic mechanisms in equilibrium. An upset of homeostasis by the sudden production of either a hormonal deficiency or an excess may result in non-specific changes which can be confused with those specifically related to the action of the hormone. The acute changes are nonetheless important to observe. In chronic studies of either hormone deficiency or excess, compensating functions of the organism may partially or completely re-establish homeostasis, and thereby mask the effects of the change in hormone intake.

#### *The Possible Role of Hyperadrenocortinism in Some of the Metabolic Adjustments During Stress*

The adrenal cortices of laboratory animals are known to hypertrophy and increase their secretory activity during stress. In at least some instances, the accompanying metabolic changes resemble those caused by the administration of either large amounts of the cortical hormones or of ACTH. Are these metabolic changes mediated by an increased secretion of hormones by the adrenal cortices? The following experiments are relative to this problem.

*Estrogen Diabetes.* Diethylstilbestrol and other estrogens are diabetogenic

in the force-fed, partially depancreatized rat.<sup>17</sup> Estrogen diabetes cannot easily be demonstrated in the rat eating *ad libitum*, for the reduction in food intake during the administration of estrogens is so great that the glycosuria may be reduced and the diabetogenic action of the hormone completely masked. Since the administration of diabetogenic amounts of estrogens is damaging to the animal and causes marked adrenal cortical hypertrophy, it seemed reasonable to postulate that the diabetogenic action is due to the increased secretion of diabetogenic adrenal steroids. Our first test of the hypothesis seemed to confirm it, for we were unable to demonstrate a diabetogenic effect of diethylstilbestrol in the adrenalectomized, partially depancreatized rat. The results of the following study<sup>18</sup> make the hypothesis untenable. Partially depancreatized rats, which were without spontaneous glycosuria, became diabetic during the injection of 0.1 mg. of diethylstilbestrol daily, and the glycosuria disappeared when the injections were stopped. The animals were then adrenalectomized and treated with a sub-diabetogenic amount (3 cc. daily) of adrenal cortex extract. Glycosuria developed when diethylstilbestrol was injected and disappeared when the injections were stopped. When the animals were maintained by treatment with either 11-desoxycorticosterone acetate or by drinking 0.9 per cent sodium chloride, the diabetogenic effect of the estrogen was either slight or absent. These data show that the presence of the cortical hormone is essential for a full manifestation of estrogen diabetes, but that an increased secretion of the cortical hormone, in response to the estrogen, is not required.

This study was extended as follows<sup>19</sup>: adrenalectomized, hypophysectomized, partially depancreatized rats were maintained on sub-diabetogenic amounts of adrenal cortical and anterior pituitary extracts. The administration of diethylstilbestrol caused hyperglycemia and glycosuria in all of the test animals which were without glycosuria during the control periods, and, in animals having spontaneous glycosuria, it became more severe when the estrogen was administered.

Diethylstilbestrol also causes an increase of liver glycogen in the intact rat, but not in the untreated adrenalectomized rat.<sup>20, 21</sup> This has been considered proof that the response is mediated by an increase in the secretion of the cortical hormones. A more critical test of the hypothesis in adrenalectomized animals maintained on a steady intake of adrenal cortical extract will be required.

#### *Effects of Other Forms of Stress on Carbohydrate Metabolism*

On the supposition that other non-specific damaging agents might be diabetogenic if they caused adrenal cortical hypertrophy and an increased secretion of its hormones, we tested the effect of water intoxication, phenol, formaldehyde, carbon tetrachloride, and cold in the mildly diabetic, partially depancreatized rat.<sup>17, 22</sup> In no case was a diabetogenic effect observed. These studies should be extended by testing other forms of stress over periods of several weeks before the conclusion is drawn that diabetes cannot be intensified by adrenal cortical activation during stress.

When normal fasting rats are subjected to low pressures, there is an increase in the glycogen content of the liver. The response disappears in the

adrenalectomized animal,<sup>23, 24</sup> but according to Langley,<sup>25</sup> if the adrenalectomized rat is sustained with adrenal cortical extract in sub-glycogenic doses, anoxia does cause a glycogenic response which is independent of change in the cortical hormone intake. Similarly, crude extracts of the anterior pituitary are diabetogenic in the partially depancreatized rat, but not in the absence of the adrenal glands. Long, Katzin, and Fry<sup>4</sup> have shown that treatment of such animals with adrenal cortical extract maintains the responsiveness to anterior pituitary extract.

*The Effect of Adrenalectomy Upon the Excretion of Nitrogen During Forced Feeding and During Fasting*

Adrenalectomy in laboratory animals is usually followed by a decrease in the excretion of non-protein nitrogen, thereby indicating a lessened ability of the animal to metabolize protein. Such changes may be due in part to a decrease in intake of food, renal failure, circulatory failure, and other secondary changes of cortical insufficiency. We have studied<sup>26</sup> the nitrogen excretion of adrenalectomized and sham-operated rats during forced feeding and during fasting. The animals were given a 1 per cent solution of sodium chloride to drink during all phases of the experiments. In the first experiment (FIGURE 8), 8 rats were adrenalectomized and 7 were sham-operated.

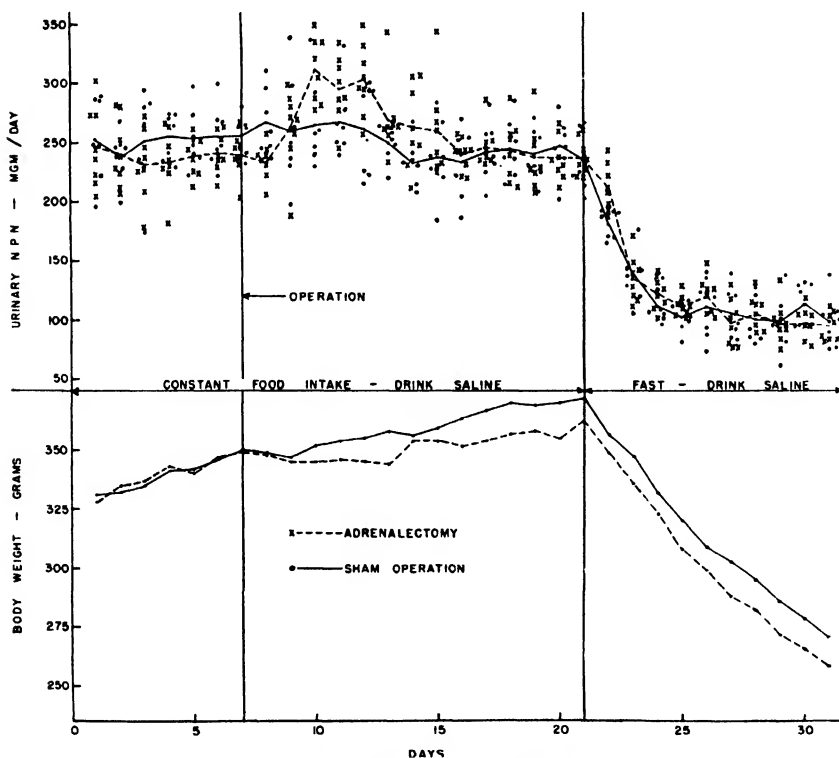


FIGURE 8. Urinary non-protein nitrogen. Averages and individual values from male rats on a medium carbohydrate diet.

During the first postoperative day, the adrenalectomized rats excreted less nitrogen, but by the third day there was a rise in urinary nitrogen which was sustained for several days and which was greater in the adrenalectomized rats than in their controls. This difference in response has been confirmed in five subsequent experiments. During the two weeks of postoperative feeding, the urinary nitrogen of the adrenalectomized group did not decrease significantly below the pre-operative level or that of the control group. During a ten-day fast, the adrenalectomized rats excreted as much urinary nitrogen as their controls. In a second study (FIGURE 9), a medium protein diet

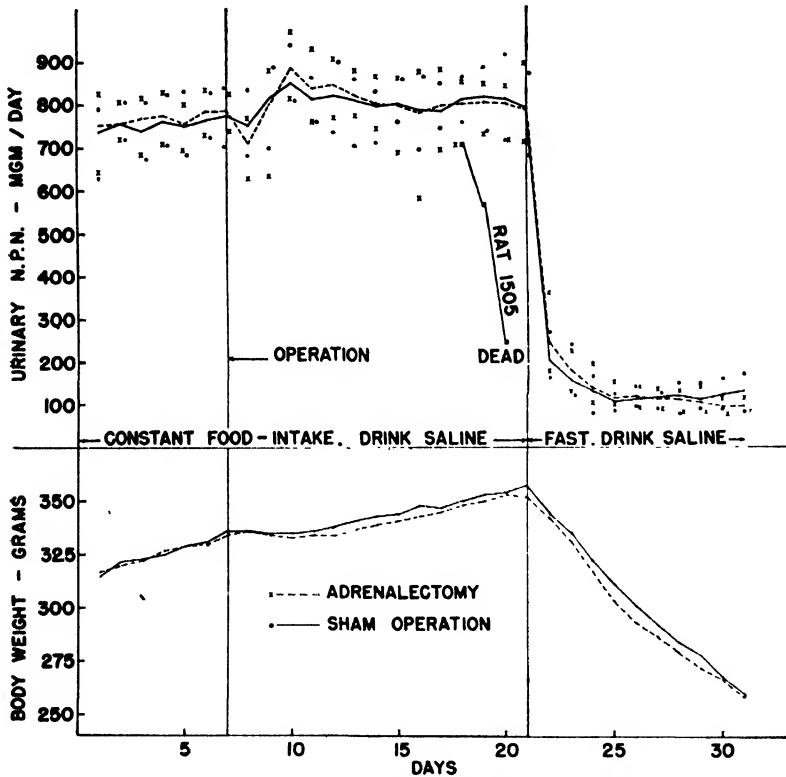


FIGURE 9. Urinary non-protein nitrogen. Averages and range of values from male rats on a medium protein diet.

was force-fed to 7 pairs of rats. The results paralleled those of the preceding experiment up to the 9th and 10th days of fasting, when the level of urinary nitrogen of the sham-operated rats was definitely higher than that of the adrenalectomized rats.

A similar experiment (unpublished) was carried out with the forced feeding of a high protein diet. The average amount of nitrogen excreted by the adrenalectomized, saline-treated animals was significantly less than was excreted by their sham-operated controls; but there was overlapping in the individual values, and amounts of non-protein nitrogen up to 1530 mgm. per

day were excreted by some of the adrenalectomized group. Data on the two longest surviving animals are shown in FIGURE 10. Symptoms of

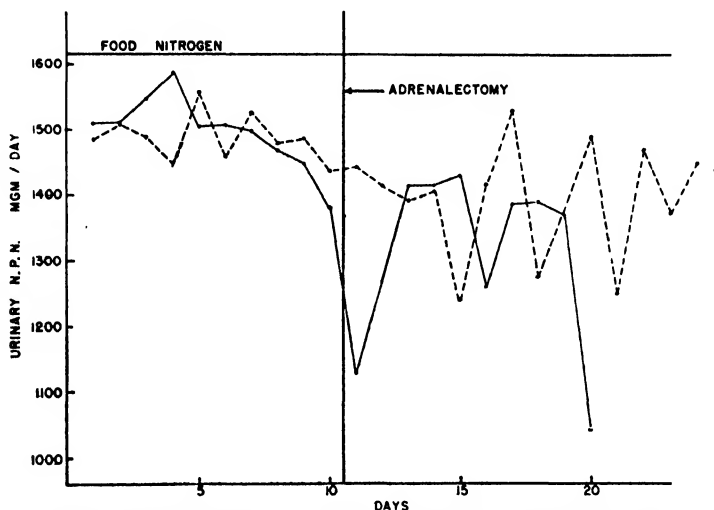


FIGURE 10. The effect of adrenalectomy upon urinary nitrogen in two male rats on a high protein diet.

cortical insufficiency were manifest within the first few days, and all of this group died within 14 days following adrenalectomy. There was failure of absorption and an eventual development of diarrhea in all of the adrenalectomized animals. In contrast, non-adrenalectomized rats can withstand considerable overfeeding of this diet, and we have observed daily values for urinary non-protein nitrogen up to 2100 mgm. before the animals were killed by overfeeding.

These and other experiments<sup>27</sup> show that it is possible for adrenalectomized rats to metabolize large amounts of fed protein when maintained in good condition on a favorable intake of sodium chloride. None of our force-fed animals showed any degree of failure until they became sick. The failure of the animal to metabolize fed protein may be secondary to a failure of supporting mechanisms, particularly circulatory mechanisms. However, the ease with which one may make this assumption should not be mistaken for proof. Nothing has been shown of the kinetics of protein catabolism or of the turnover of amino acids between the body fluids and tissues, and it has not been shown that the adrenalectomized, saline-treated rat can tolerate as great a dietary load as the non-adrenalectomized rat. The question is unsettled.

Is the adrenalectomized animal able to mobilize its body proteins as well as does the intact animal? Although a number of studies<sup>4</sup> have shown that it does not, there was little evidence that our adrenalectomized rats differed from normal in their excretion of non-protein nitrogen during fasting. Our studies differed from earlier acute experiments in that adrenalectomized rats were maintained for two weeks by forced-feeding diets relatively low in carbohydrate prior to fasting. The fasting rat has a very low level of nitro-



gen excretion as compared to other animals, but it can mobilize its own tissues very rapidly during stress. In order to test fully the ability of the adrenally insufficient animal to mobilize its own tissues, the comparison should be made under conditions where the rate of mobilization is stimulated in the intact animal. Some such studies are to be described.

*The Effect of Fractures on Nitrogen Excretion in Adrenalectomized and Sham-Operated Rats*

In the first experiment (FIGURE 11), 12 rats were maintained on the

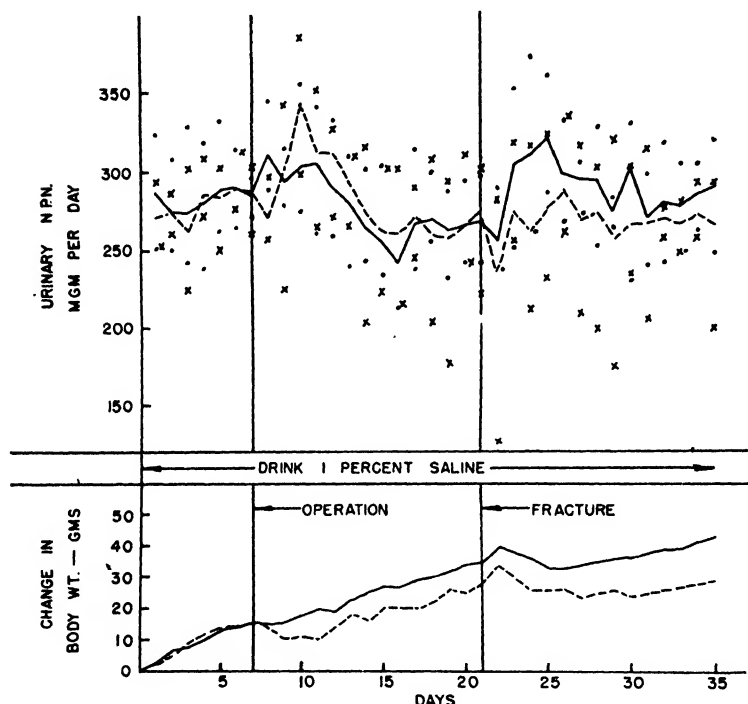


FIGURE 11. The effect of fractures upon urinary N.P.N. in adrenalectomized and sham-operated male rats. Averages and range of values.

medium carbohydrate diet for two weeks, at which time 6 rats were adrenalectomized and 6 were sham-operated. All of the rats were given 0.9 per cent sodium chloride to drink during the entire experiment. No hormone was given in this experiment. Two weeks following operation all of the animals were subjected to fracture of the knee-joint and tibia of the right hind leg. There was a rise in nitrogen excretion following operation in each group, again higher in the adrenalectomized rats; but subsequently there was no significant rise following fractures in the adrenalectomized animals such as occurred in the non-adrenalectomized rat.

Recalling the results of our experiments<sup>18</sup> on the diabetogenic activity of diethylstilbestrol in adrenalectomized rats, the next step was to substitute

a constant intake of adrenal cortex extract for the adrenal glands and to test again the response to fractures. Accordingly, in the second experiment (FIGURE 12), treatment with saline was omitted and the adrenalectomized

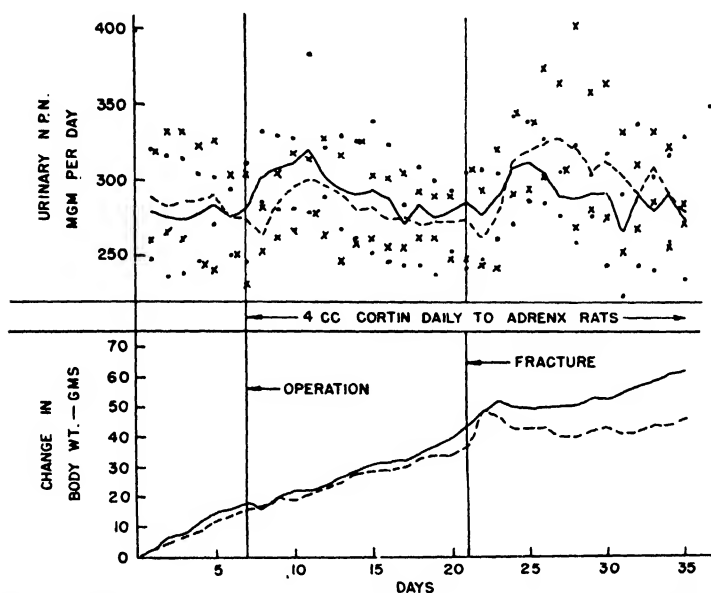


FIGURE 12. The effect of fractures upon urinary N.P.N. in adrenalectomized rats treated with cortica extract and sham-operated male rats. Averages and range of values.

rats were treated with 4 cc. daily of beef adrenal extract, from the time of operation throughout the following four weeks. The rise in nitrogen excretion following operation was less in the cortin-treated animals than in their sham-operated controls and less than was shown by the saline-treated, adrenalectomized animals of the preceding experiment (FIGURE 9). Additional study will be required to determine whether there is a true difference in the response of saline-treated and cortin-treated rats in respect to nitrogen loss following adrenalectomy. Following the fracturing of the tibia and knee-joint, the adrenalectomized, cortin-treated rats excreted less nitrogen during the first 24 hours, but thereafter showed a rise in nitrogen excretion which was even greater than that of the control series.

In the third experiment (FIGURE 13), the conditions were identical with those of the preceding study, except that the tibia, femur, and knee-joint of both hind legs were fractured in each rat. The results were similar to those of the preceding study, except that the post-fracture rise in nitrogen excretion was greater in both the adrenalectomized and non-adrenalectomized animals.

Here is an example of the subnormal ability of the untreated adrenalectomized animal to mobilize its tissue proteins following damage. Does this reflect a primary deficiency of some prepotent function of the cortical hormones? The adrenalectomized animals of this study appeared to be

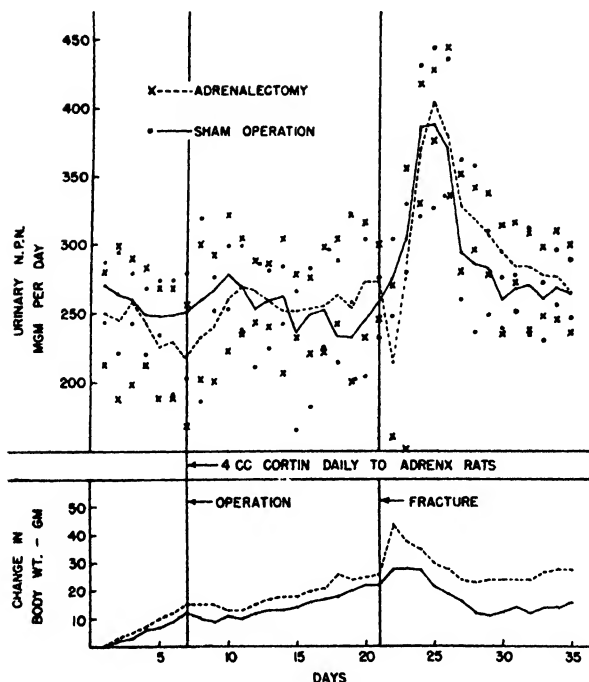


FIGURE 13. The effect of extensive fractures upon urinary N.P.N. in adrenalectomized rats treated with adrenal cortex extract and sham-operated male rats. Averages and range of values.

mildly "sick" following the fractures. Did we deal with a primary or a secondary deficiency? We do not know. The hypothesis, that the post-fracture rise in nitrogen is due specifically and solely to an increased secretion of the cortical hormones following stress, is clearly incorrect. The presence of the hormones was required to maintain the responsiveness of the tissues, but an increase in hormone was not the only cause of the response.

In all of our experiments, there has been a difference in the nitrogen excretion of non-adrenalectomized rats and either cortin- or saline-treated, adrenalectomized rats during the first 24 hours following an operation or a fracture. This either may reflect a difference in the dissolution of lymphoid tissue,<sup>28</sup> which does seem to occur rapidly and extensively only in response to increased adrenal cortical activity, or it may reflect secondary factors.

*The Effect of Burns on Nitrogen Excretion in Adrenalectomized and Cortin-Treated Rats.* A rise in the excretion of urinary nitrogen is a typical response to an extensive burn. As shown in FIGURE 14, this response occurs in adrenalectomized, cortin-treated rats just as in the sham-operated controls and was therefore independent of any change in adrenal activity. Among four adrenalectomized rats treated with saline, only two animals showed a marked negative nitrogen balance following a burn, and two failed to show a significant increase in urinary nitrogen, but did manifest diarrhea and weakness.

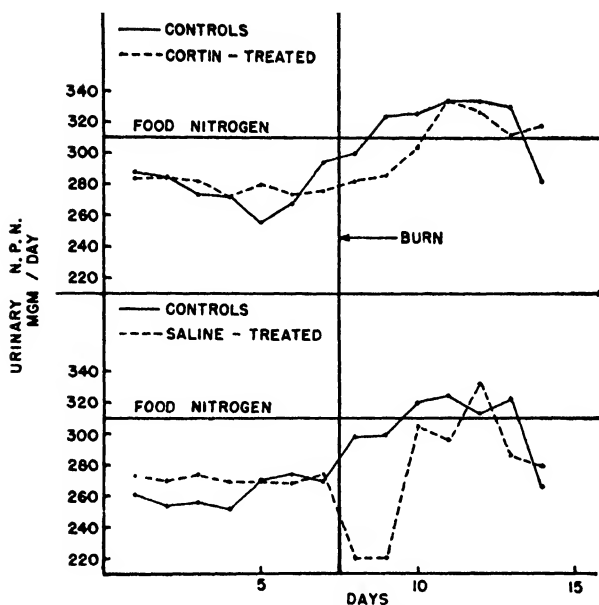


FIGURE 14. The effect of burns upon urinary N.P.N. in adrenalectomized rats, with and without cortical hormone, and in control rats. Averages.

*The Effect of Thyroxin Upon Nitrogen Balance in Adrenalectomized Rats.* It can be argued that the nitrogen lost following an extensive fracture or a burn may arise from the autolysis of damaged tissue and that is not mobilized by physiological mechanisms. This may or may not explain all of the loss of tissue protein following tissue injury; nevertheless, the animal is able to catabolize it to nonprotein compounds, for the nitrogen is excreted as such. In an effort to test the ability of the adrenalectomized rat to catabolize its own tissues by means of non-pathological mechanisms, thyroxin was administered to eight adrenalectomized, saline-treated rats. Two of these animals showed a striking response, as is illustrated by the data on one rat in FIGURE 15. Although all of them showed an increase in nitrogen loss, some developed symptoms of adrenal cortical insufficiency and died without showing a normal response. The effect of thyroxin in the cortin-treated, adrenalectomized animal has not been studied by us. A number of years ago, a highly important study of the effect of thyroxin in the partially adrenalectomized dog was made by Koelsche and Kendall.<sup>29</sup> In brief, it was shown that the partially adrenalectomized dog was more easily thrown into negative nitrogen balance by the administration of thyroxin than the normal dog and that treatment of the partially adrenalectomized dog with cortical extract raised the resistance of the animal to thyroxin and restored a positive nitrogen balance.

### Discussion

It is abundantly evident from our own and other studies that the adrenal cortex is not the sole regulator of protein breakdown and that other adaptive

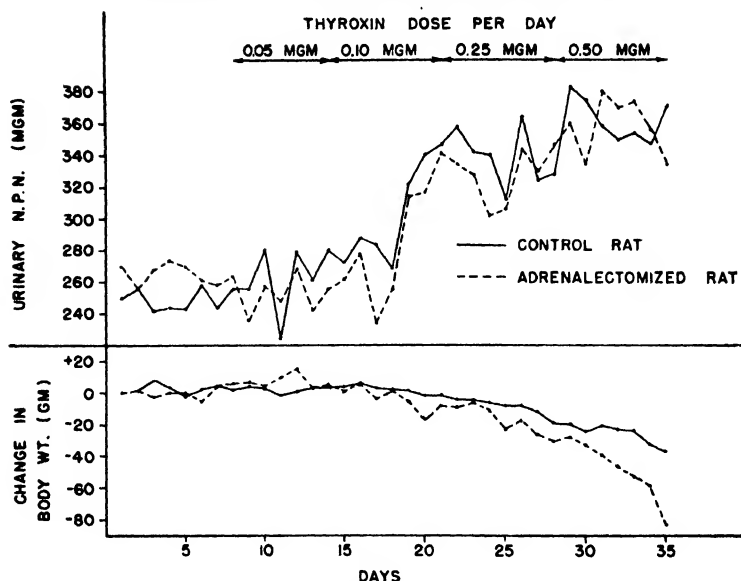


FIGURE 15. The effect of thyroxine upon urinary N.P.N. in one adrenalectomized and one control rat.

mechanisms can operate in the absence of any change in the intake of the cortical hormones. This is in keeping with established principles of physiology. In the entire field of physiology, I do not know of any metabolic process which is exclusively the function of any single organ. On the other hand, the cortical hormones can indeed affect the metabolism of protein, for there are the well-established effects of the C-11-oxygenated steroids in causing the breakdown of lymphoid and other tissues and in inhibiting growth. Albright<sup>30</sup> has offered the attractive hypothesis that the effect of the cortical hormones upon the structural proteins and growth may represent a blocking of protein synthesis, an "anti-anabolic" rather than "catabolic" effect. The breakdown of lymphoid tissue is too rapid to be explained on such a basis and must reflect, at least in part, a "catabolic" action. The nature of the effect on structural tissues is less certain.

The production of hyperadrenocorticism by the administration of either the cortical hormones or ACTH is characterized by a diabetogenic effect in the rat and a negative nitrogen balance. Why are not these same metabolic changes caused by adrenal cortical hyperfunction when it is stimulated by stress? It has been suggested<sup>18</sup> that the increased secretion of the cortical hormones to meet an increased need for their function does not cause over-dosage phenomena but serves only to maintain homeostasis. It is certain, at least, that the response of the adrenal cortex during stress serves some purpose which is essential for normal resistance.

The data of these studies emphasize the inhibitory effect of the cortical hormones upon carbohydrate utilization rather than its positive effect upon gluconeogenesis. But how does inhibition of carbohydrate utilization relate to the effect of these hormones upon body economy? Of what use is a

"diabetogenic" action? Adrenal steroid diabetes is an overdosage phenomenon, but it may represent an exaggeration of normal function. Possibly, it favors the oxidation of fat and conserves carbohydrate for emergency use during a stress or during fasting.

The brilliant studies of Stetten<sup>31, 32</sup> indicate that the conversion of carbohydrate to fat is a normal and important pathway in its utilization and that one of the principal defects in diabetes is the inability of the animal to convert carbohydrate to fat. In studies on the overfeeding of a high carbohydrate diet, we<sup>33</sup> have observed that normal rats can tolerate enormous amounts of fed carbohydrate by storing it as fat. Why cannot animals having either pancreatic or adrenal steroid diabetes dispose of the excess blood glucose by converting it to fat? They do not conserve glucose in either case, but waste it by excretion into the urine. In adrenal steroid diabetes, there may also be an inability of the animal to convert its carbohydrate to fat. To the contrary, there is some evidence that the cortical hormones favor the storage of fat.<sup>34, 35</sup>

How are the various effects of the cortical hormones upon organic metabolism related one to the other? In this paper, I have described an unsatisfactory attempt to explain the stimulation of gluconeogenesis as a compensatory response to the inhibitory action upon the utilization of carbohydrate. The studies of Price *et al.*<sup>36</sup> have shown that certain adrenal cortical principles modify the hexokinase reaction by intensifying the inhibitory effect of anterior pituitary extract upon the action of insulin. Stetten<sup>37</sup> has suggested that the position of the hexokinase reaction among the processes of carbohydrate utilization would permit a possible explanation of the effects of the adrenal cortical hormones upon the formation, storage, conversion, and oxidation of carbohydrate. There are at least two arguments against this hypothesis: First, the adrenal hormones exert a diabetogenic and glycogenic action in the hypophysectomized animal, whereas, in the studies of Price *et al.*,<sup>36</sup> the cortical hormones did not affect the hexokinase reaction in the absence of anterior pituitary extract. Second, Price *et al.* obtained marked effects with the amorphous fraction of cortical extracts and little or no effect with the compounds 11-dehydrocorticosterone, corticosterone, and 17-hydroxy-11-dehydrocorticosterone which are active in the intact animal. It is possible to explain these discrepancies by assuming that the hypophysectomized rat has a trace of either anterior pituitary hormone or anterior pituitary-like hormone which synergizes the action of the cortical hormone and, secondly, that the C-11-oxygenated steroids failed to show activity in the *in vitro* experiments because of their insolubility.

The thinking of many of us in the adrenal field has become too rigidly channelized during the past few years. It is common to describe adrenal physiology in terms of electrolyte and organic (generally limited to protein and carbohydrate) metabolism as though they are completely unrelated functions, electrolyte metabolism being controlled by 11-desoxycorticosterone and organic metabolism being controlled by the C-11-oxygenated steroids. The story is not this simple. It is true that 11-desoxycorticos-

terone has its most striking effect upon electrolyte balance and that only the C-11-oxygenated compounds are potent in their effects upon carbohydrate metabolism. But there is considerable overlapping of effects, and there is a great deal of evidence that there are basic relationships between the distribution of inorganic ions and the metabolism of organic compounds which relate to adrenal cortical functions.

We in the adrenal field have been observing the end results of cortical hormone deficiency and cortical hormone action, and have spoken of end results as though they were the processes of action. We know none of the reactions into which the cortical hormones enter to bring about their metabolic effects. The metabolic changes which result from cortical insufficiency and excess are nonetheless important to consider. The correction of sodium loss and carbohydrate deficiency in cortical insufficiency may determine whether the animal or patient is able to live. But, not all of the effects of the cortical hormones upon the viability and vigor of the animal can be defined in terms of what is now known of the metabolic effects of these hormones. In the non-adrenalectomized animal, the sodium and chloride content of the blood can be depleted to levels lower than occur in adrenal cortical insufficiency without causing death. Similarly, lower values for blood glucose and tissue glycogen can be tolerated by non-adrenalectomized animals. Non-adrenalectomized animals can tolerate higher levels of serum potassium than usually occur in cortical insufficiency. Many of the symptoms of cortical insufficiency resemble those of animals dying of other causes. Other steroid compounds, particularly estrogens, have a corticoid action in that they cause sodium retention and exert a diabetogenic action without any favorable effect upon the survival and vigor of the adrenalectomized animal.

The most important and remarkable advances in the adrenal field have been the isolation, identification, and synthesis of several biologically active compounds. The practical advances in the treatment of adrenal disease and many of the advances in physiology awaited the contributions of the chemist. Now the new techniques of enzymology and isotope chemistry may be the tools required to probe into the processes by which the cortical hormones produce their effects. In addition to techniques, new and broader concepts are needed. I hope that there will remain a place for physiologists who can advance the methods of experimenting upon animals and who will, at appropriate intervals, remind the chemist that the organism functions as a whole.

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# THE CYTOLOGY AND CYTOCHEMISTRY OF THE ADRENAL CORTEX\*

By ROY O. GREEP AND HELEN WENDLER DEANE

*Harvard School of Dental Medicine and Department of Anatomy, Harvard Medical School, Boston, Massachusetts*

The present paper concerns itself with the cytological and cytochemical aspects of the adrenal cortex. We have endeavored to interpret whatever morphological and chemical changes have been detected in terms of the demand for and the evidence of hormone production. The adrenal cortex is exceptionally well adapted for cytophysiological and cytochemical study because clear-cut alterations in its chemical and structural make-up follow quickly any disturbance in the bodily equilibria with which the cortex is concerned. Moreover, considering that high local concentrations of compounds are an important requisite to a cytochemical study, the cortical constituents which fulfill this requirement are triglycerides, steroids and ascorbic acid.

## *Histology of the Adrenal Cortex*

The functional elements, or parenchyma, of the cortex are large polygonal epithelial cells. These cells comprise the great bulk of the tissue. On the basis of differences in the arrangement of the parenchymal cells, Arnold divided the cortex into concentric layers which, from the periphery inward, are named: the *zona glomerulosa*, made up of loops and balls of cells; the *zona fasciculata*, having long columns of cells; and the *zona reticularis*, in which the fascicular columns become distorted and broken.<sup>2</sup> The fasciculata has been subdivided further on the basis of the lipid droplets contained within its cells into a thin subglomerular region referred to variously as the transitional, lipid-free, or "sudanophobic"<sup>60</sup> zone, a broad and very fatty outer fasciculata containing the so-called spongiocytes, and an equally broad inner zone in which the lipid droplets of the cells are smaller and less numerous.

The cortex has an extremely rich vascular bed.<sup>3</sup> Arterioles enter from the capsule and immediately break up into numerous long sinusoids, which run radially between the columns of cells (FIGURE 1). The cells near the capsule are therefore more advantageously situated with respect to a fresh supply of oxygenated blood. Parenthetically, it may be remarked that the adrenal cortex appears to lack innervation and is, therefore, probably entirely under hormonal control. The connective tissue stroma consists of a reticulum of argyrophil fibers which underlies the sinusoids and enmeshes the cells.

## *Cytology of the Adrenal Cortex*

Under the present heading, we shall consider the mitochondria, or chondriosomes, the Golgi apparatus, and the lipid droplets. The first two of

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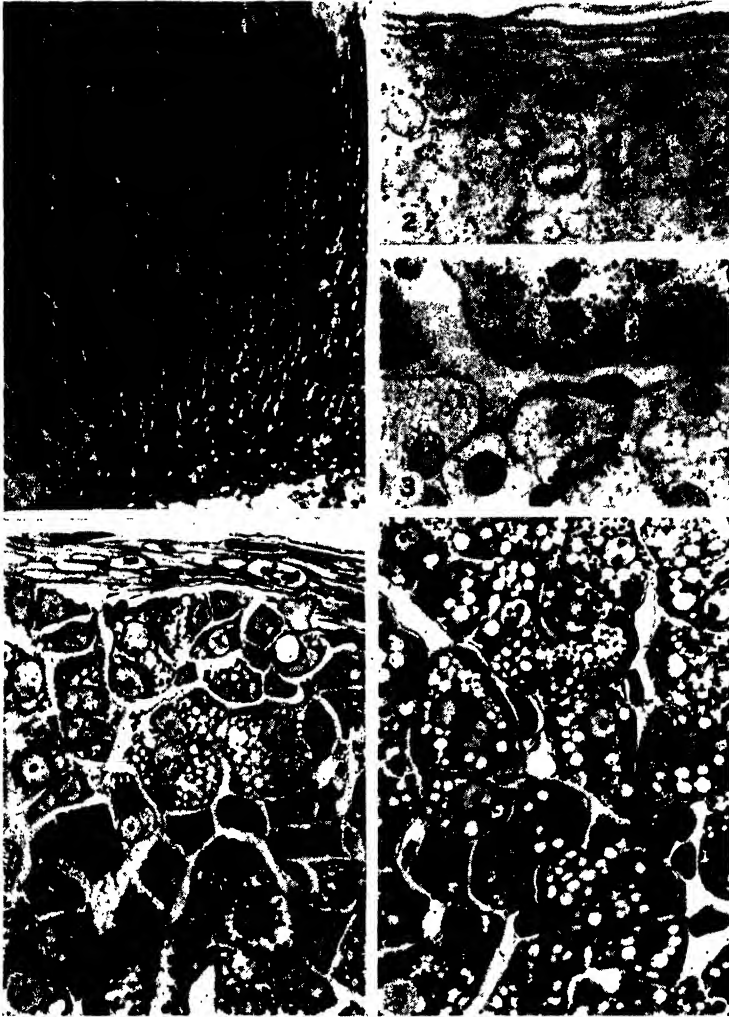
these structures are the subject of an extensive literature for which comprehensive reviews are available,<sup>2, 61, 63</sup> so that there appears to be no point in discussing them here, beyond examining their usefulness for characterizing the various zones of the cortex and noting whatever changes may occur in different states of activity. A valuable recent review concerning the general nature and activity of the mitochondria and Golgi apparatus is to be found in Bourne's *Cytology and Cell Physiology*.<sup>8</sup>

### *Mitochondria*

With improved techniques, the tendency in recent years has been to demonstrate fewer and fewer bizarre forms of chondriosomes in the cortical cells; the rings, beads, clubs, etc. have largely disappeared from descriptions. At present, the mitochondria of the cortex of normal animals are observed to be either spherical granules or short rods, depending on the species. Pertinent studies are available for the guinea pig,<sup>35</sup> cat,<sup>2</sup> and rat.<sup>16</sup>

The cells of the glomerulosa have an exceedingly dense population of mitochondria, which in the guinea pig and cat are rod shaped, but which in the rat are small, uniform spherules. In the guinea pig and cat, they are distributed quite evenly throughout the cytoplasm, thus giving these cells, which have relatively few fat vacuoles, a homogeneous appearance. In the rat, where the glomerulosa contains more numerous lipid droplets, they lie in the interstices between the droplets (FIGURE 4). In the cells of the fasciculata, the mitochondria are spherical in all the species which have been studied, and occur in the protoplasmic films between the lipid droplets. In the rat, the only morphological difference which we have noted between these two major zones is that the mitochondrial spherules are larger in the fasciculata than in the glomerulosa (FIGURE 5). The reticularis is characterized mainly by an extraordinary variability in the size and number of the chondriosomes. In some cells they are large, in others small, but within any given cell the size is generally regular. In this zone, occasional "light" cells have only a few mitochondria and contrast sharply with "dark" cells in which the mitochondria are so densely packed that it is difficult to recognize other structures in the cytoplasm. This difficulty is enhanced by the fact that the cytoplasm of the "dark" cells stains intensely with iron-alum hematoxylin. Because of this property, the "dark" cells are frequently called siderophiles. Furthermore, in some cells of the reticularis the mitochondria are fragmented, stain poorly, and are distributed irregularly.<sup>2</sup> These phenomena are ordinarily associated with cell degeneration.

We have examined the mitochondria in the various zones of the rat adrenal following hypophysectomy,<sup>16</sup> and Deane and co-workers<sup>17, 19</sup> have made similar studies on rats in which the adrenals had been stimulated to hyperactivity. Hypophysectomy does not alter the mitochondria in the glomerular zone, but in the spongy or outer layer of the fasciculata their size is markedly reduced, so that they come to resemble those of the glomerulosa.<sup>16</sup> In rats subjected to deficiencies of pantothenic acid<sup>17</sup> or of thiamine,<sup>19</sup> in both of which there is evidence of hyperactivity of the zona fasciculata, the mitochondria of this zone are enlarged and become less chromophilic. When



FIGURES 1-5

FIGURE 1. Section of a cat's adrenal which had been injected with dilute India ink via both the aorta and the vena cava so as to visualize the sinusoids. The sinusoids can be seen outlining the glomerulosa and then paralleling the cell columns of the zona fasciculata toward the medulla. The reticularis is visible in the lower part of the figure. The sinusoids are particularly narrow in the outer portion of the fasciculata. From BENNETT & KILHAM.<sup>1</sup> X 58.

(FIGURES 2-5 are drawings with the aid of a camera lucida, made by Etta Piotti.)

FIGURE 2. Rat's adrenal cortex treated with acid silver nitrate to demonstrate ascorbic acid. Capsule and zona glomerulosa. Small granules of reduced silver are present in the cytoplasm of the glomerulosa cells and also in the inter-cellular spaces (probably the sinusoids). DEANE & MORSE.<sup>12</sup> X 1180.

FIGURE 3. Ascorbic acid in the zona reticularis (above) and the medulla of rat's adrenal, prepared as FIGURE 2. Large granules in the cytoplasm of the cells of the reticularis. Fine precipitate in the medullary cells; a macrophage shows an especial concentration of ascorbic acid. DEANE & MORSE.<sup>12</sup> X 1180.

FIGURE 4. Mitochondrial preparation of rat's adrenal cortex. Fixed in Zenker-formalin, stained with iron-alum hematoxylin. Shows capsule, zona glomerulosa, transitional zone (containing no lipid spaces), and a few cells of the outer fasciculata. The mitochondria in the cells of the glomerulosa are small spheres, those of the fasciculata somewhat larger spheres. DEANE & GREEP.<sup>13</sup> X 1180.

FIGURE 5. Mitochondrial preparation of rat's adrenal cortex, showing the middle part of the fasciculata. The cells contain spherical mitochondria (gray), large liposomes (black), and numerous lipid spaces. The mitochondria are somewhat larger than those of the glomerulosa. DEANE & GREEP.<sup>13</sup> X 1180.

the dietary deficiencies exceeded toleration, the mitochondria become vesiculated.

These experimental studies suggest that the mitochondrial form is a useful measure of cell function despite the fact that these organelles cannot be directly related to the secretory products of the adrenal cortex. Furthermore, as in other tissues, the mitochondria serve as an index of cell viability, since, when they are irregular in size and non-chromophilic, it can be assumed that the cell is dying.

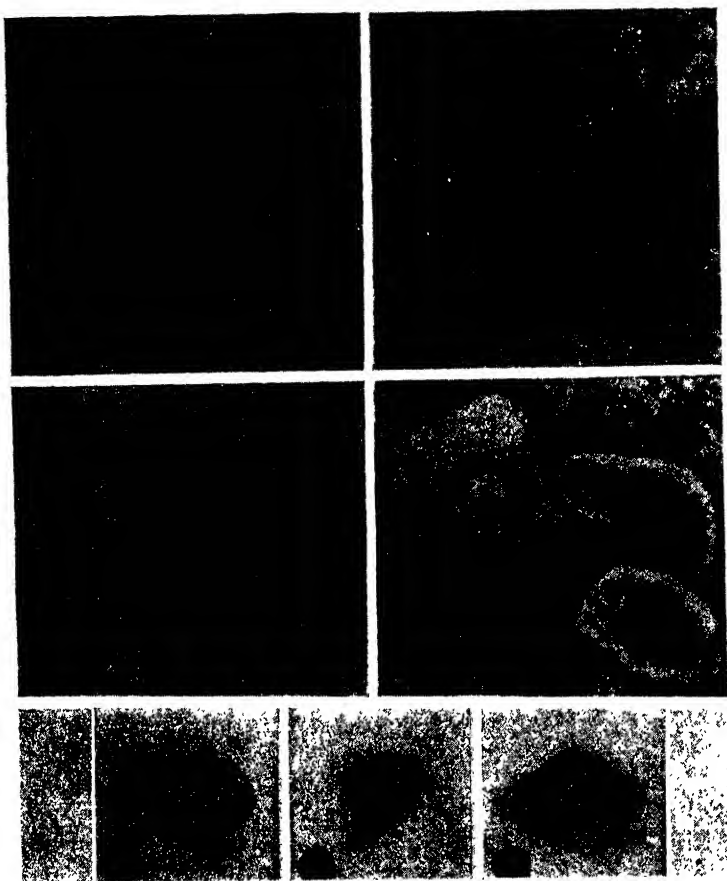
### *The Golgi Apparatus*

The Golgi apparatus in the cells of the adrenal cortex is most successfully demonstrated by the Da Fano silver method because of the large amount of fat which masks the apparatus in osmic acid preparations. By the use of the silver method, the Golgi apparatus can be shown to be a constant component of all the cells of the adrenal cortex. Minor differences are found in the form and appearance of this organelle in different species, but its location is always juxtanuclear.

Bennett<sup>2</sup> described the appearance of the apparatus in the cortex of the cat's adrenal as a network which is compactly arranged in the cells of the zona glomerulosa (FIGURE 6), loose and ramifying in the spongiocytes (FIGURE 7), compact and less well defined in the inner fasciculata (FIGURE 8), and dense and fragmenting in the reticularis (FIGURE 9). He also found that in the glomerulosa of the cat the Golgi substance is constantly located between the nucleus and the nearest capillary, thereby conferring secretory polarity on these cells. Such an orientation, however, was not noticed by Hoerr for the guinea pig,<sup>35</sup> nor by Reese and Moon for the rat.<sup>49</sup> In the rat, Bourne<sup>6</sup> observed two distinct types of Golgi net in the cortical cells—the compact and the diffuse. He believed that the latter represented an hypertrophy of the former, but he failed to describe their zonal location. Bennett, however, felt that the radial extension of the network observable in the spongiocytes is not a true hypertrophy, but rather, a dispersion caused by the presence of crowded lipid droplets.

The observations of Reese and Moon<sup>49</sup> are especially useful in that they describe the Golgi net of rats' adrenals in widely different states of secretory activity. Their observations on normal control rats are in fairly good agreement with those of Bourne. Despite great variation in the form of the Golgi substance, it is clearly most dispersed in the spongiocytes (FIGURE 10). Moreover, the apparatus is smaller and more circumscribed in the cells of the reticularis than in those of the outer fasciculata and glomerulosa. Following hypophysectomy, the Golgi apparatus shrinks and loses most of its radial extensions (FIGURE 11). This response is most marked in the spongiocytes. At only 24 hours after operation, a retraction of the extended processes is evident.

Injections of adrenocorticotrophic hormone are reported<sup>49</sup> to produce a striking hypertrophy of the Golgi apparatus, particularly in the cells of the outer fasciculata (FIGURE 12). Heavy, ramifying strands develop, and the central knot becomes much less distinct. An increase in total mass of Golgi



FIGURES 6-12

Drawings of Da Fano silver preparations for the Golgi apparatus, which appears as a blackened knot or network next to the nucleus in cortical cells. FIGURES 6-9, cat's adrenal cortex from Bennett<sup>2</sup>; drawings by Etta Piotti; X 1300. FIGURES 10-12, cells from the zona fasciculata of adrenal cortices of rats; drawings from Reese & Moon.<sup>48</sup>

FIGURE 6. Zona glomerulosa. The blackened channels which comprise the Golgi apparatus are seen as a juxtanuclear cap, lying in each cell between the nucleus and the nearest capillary.

FIGURE 7. Outer fasciculata. The Golgi apparatus sends projections and branches between the lipid "vacuoles."

FIGURE 8. Inner fasciculata. The Golgi apparatus is more compact than in the outer fasciculata, apparently because the lipid spaces are less prominent and the cells less swollen.

FIGURE 9. Zona reticularis. Fragmentation of the Golgi substance and the loss of precise impregnation are to be noted, and is interpreted as manifestations of cell degeneration.

FIGURE 10. Cell from cortex of normal immature rat. The Golgi apparatus appears as a juxtanuclear network with extensions over the nuclear surface.

FIGURE 11. Cell from a rat hypophysectomized 377 days. The cell is much smaller than that illustrated in FIGURE 10, and the Golgi apparatus appears as a shrunken and fused juxtanuclear mass.

FIGURE 12. Cell from an intact rat after the administration of 2 Moon units of adrenotropin. The cell is hypertrophied in comparison to normal, and, likewise, the Golgi net is enlarged and extended.

material is claimed. No significant change is seen in the Golgi body in the inner zones of the cortex. Furthermore, animals that receive injections of adrenotropin for 7 days following removal of the pituitary show that the hormone completely prevents the typical postoperative regression of the Golgi body.

In conclusion, it can therefore be said that the form of the Golgi net is a direct index of the secretory activity of adrenal cortical cells, being compact during inactivity and diffuse and ramifying during marked activity. In addition, most writers believe it is also larger in the spongiocytes than elsewhere.

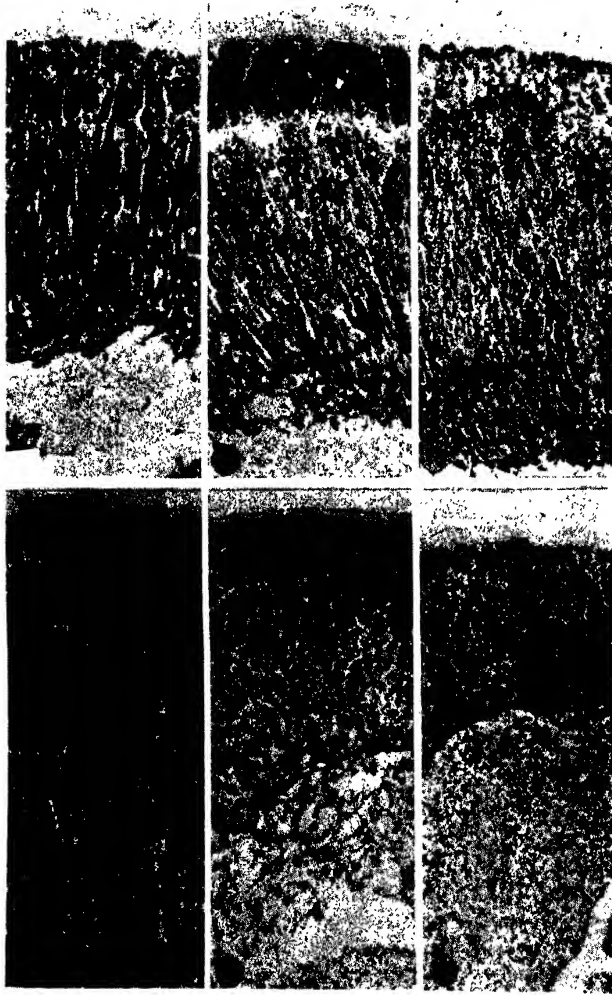
### *Lipid Droplets*

The most prominent cytological feature of the adrenal cortical cells is the presence in the cytoplasm of crowded droplets of fatty material. The lipid droplets are discrete and can be seen in fresh, unfixed tissue. In the unfixed condition, they are spherical and apparently homogeneous, and other shapes which are assumed in fixed material are probably artifacts. The droplets are soluble in organic solvents and, hence, appear as vacuoles after ordinary histological procedures which involve dehydration. It is a common observation, however, that after prolonged treatment with potassium bichromate some of these fatty droplets are preserved and stain in much the same manner as do the mitochondria.<sup>16, 36</sup> Hence, it may be assumed that these particular droplets are closely related to the mitochondria in chemical make-up, *i.e.*, contain phosphatides<sup>36</sup> or are lipoproteins.<sup>8</sup> We will restrict our use of the term "liposome" to refer to droplets which have this staining property. They differ from the simple lipid ones, from which the contents are completely dissolved in mitochondrial preparations (FIGURE 5).

The lipid droplets can be identified in frozen sections by the fact that they take up the fat-soluble, or sudan, dyes. This is a precise cytochemical test. Contrary to general opinion, however, the depth of color of the droplet with the red sudans cannot be related to the chemical constitution thereof, but is attributable to the physical factor of solubility.<sup>43</sup> The lipid droplets also reduce osmium tetroxide, yielding a black product. This is not a specific test for fats, since any unsaturated compound may reduce osmic acid. The use of sudan stains is therefore to be preferred.

In Bennett's study of the cat's adrenal,<sup>2</sup> the locations of the osmiophilic and the sudanophilic substances were essentially identical. The cells of the outer fasciculata, or what he termed the secretory zone, were packed with fairly even-sized droplets, a few of which could be seen outside the cells and apparently entering the sinusoids. None of the other zones of the cat's adrenal were described by him as containing more than a sparse amount of lipid, except for some cells in the reticularis that contained a considerable number of irregular-sized droplets. His illustrations, however, show a sudanophilic outer zone (glomerulosa), which is set off from the lipid-rich fasciculata by a distinct transitional zone (his Figures 4 and 7). We have found that the glomerulosa of the dog's adrenal is conspicuously sudanophilic in comparison to that of the cat (FIGURE 13).

The rat's adrenal, which has been the subject of much study from the standpoint of the distribution of osmiophilic or sudanophilic material,<sup>12, 13, 17, 21, 24, 48, 52, 60</sup> has a very fatty glomerulosa. The lipid droplets in this zone are somewhat larger than those in the fasciculata and are more intensely sudanophilic. The lipid-free zone interposed between these two



FIGURES 13-18

The photomicrographs of FIGURES 13-24 are of  $15\mu$  frozen sections of formalin-fixed adrenals. FIGURES 13-15 are of adrenal cortices of dogs, stained with sudan IV for lipids, no counterstain. From McKIRBIN & DEANE.<sup>44</sup>  $\times 75$ . FIGURES 16-18, adrenal cortices of rats stained with the Schiff reagent. Ketosteroids can react the Schiff reagent and produce a purple stain. The border between the cortex and the medulla has been drawn in. From GREEP & DEANE.<sup>51</sup>  $\times 135$ .

FIGURE 13. Cortex of a normal dog, showing three major zones: a conspicuous glomerulosa; a broad fasciculata; and a narrow juxtamedullary reticularis. Sudanophilic lipids are abundant throughout the cortex, being somewhat less so in the transitional zone between the glomerulosa and fasciculata.

FIGURE 14. Cortex of a dog on a pantothenic acid deficient diet for a month and suffering from a severe deficiency—not moribund, however. A marked deficiency of pantothenic acid induces the adaptation syndrome.<sup>17</sup> Severe depletion of sudanophilic lipids has occurred from the inner zones, with some lipid remaining in the outer fasciculata; a normal or supranormal amount of lipid remains in the glomerulosa. This illustrates the "near exhaustion" stage of the adaptation syndrome.

FIGURE 15. Cortex of a dog on a pantothenic acid-deficient diet for about 3 weeks, killed when in coma.

major zones is normally a prominent feature.<sup>54, 60</sup> In the outer fasciculata, the lipid droplets are extremely numerous, whereas, in the inner fasciculata, they are distinctly less numerous and perhaps somewhat smaller in size than in the outer part. The reticularis has droplets which vary enormously in size and shape. They are probably products of fatty degeneration since, as will be shown later, they fail to present the more specific ketosteroid reactions. It should be remarked here that several investigators<sup>13, 54, 57</sup> have described the lipid droplets of the zona fasciculata as fine when the gland is in a state of activity and much coarser during inactivity.

### *Cytochemistry of the Adrenal Cortex*

#### *Reactions for Ketosteroids*

The point of greatest interest concerning the lipid droplets is the fact that the adrenal hormones, being lipid-soluble ketosteroids, might be expected to be found dissolved in them. Some of the chemical methods which are used to identify ketosteroids have been adapted to use on tissue sections. Bennett,<sup>2</sup> pioneering in the field of histochemistry applied to ketosteroids, showed that acetone-soluble material present in the adrenal cortex reacts in a manner similar to ketosteroids *in vitro*.

There are, at present, the following cytochemical tests which are significant in identifying ketosteroids in tissues, and, hence, are available for localizing the biologically active hormones of the adrenal cortex.

*Phenylhydrazine reaction,<sup>2</sup> Schiff reaction,<sup>23</sup> and semicarbazide reaction.<sup>2</sup>* These three tests all depend on the presence of a ketone or carbonyl group ( $C=O$ ) in the reactive molecule. Such a group will react with phenylhydrazine to form yellow phenylhydrazones, with leucofuchsin to form a magenta-colored complex (the Schiff or "plasmal" reaction), and with semicarbazide to form semicarbazones. The latter reaction is virtually colorless and is used cytochemically only to prevent other ketone reactions. Ketosteroids, since they possess one or more carbonyl groups, are capable of giving these three tests. It is the lipid droplets of the glomerulosa and the fasciculata which react most strongly with the Schiff reagent (FIGURE 16), although the cytoplasm stains diffusely to a slight degree.

*Reichstein's ammoniacal silver reaction.<sup>2, 50</sup>* Some ketosteroids possess carbonyl groups active enough to reduce ammoniacal silver solutions. This property is exhibited particularly by the adrenal steroids which possess a carbonyl group at  $C_{20}$  and an adjacent hydroxyl at  $C_{21}$ . In tissue sections, this reaction is abolished by previous treatment with semicarbazide.<sup>2</sup>

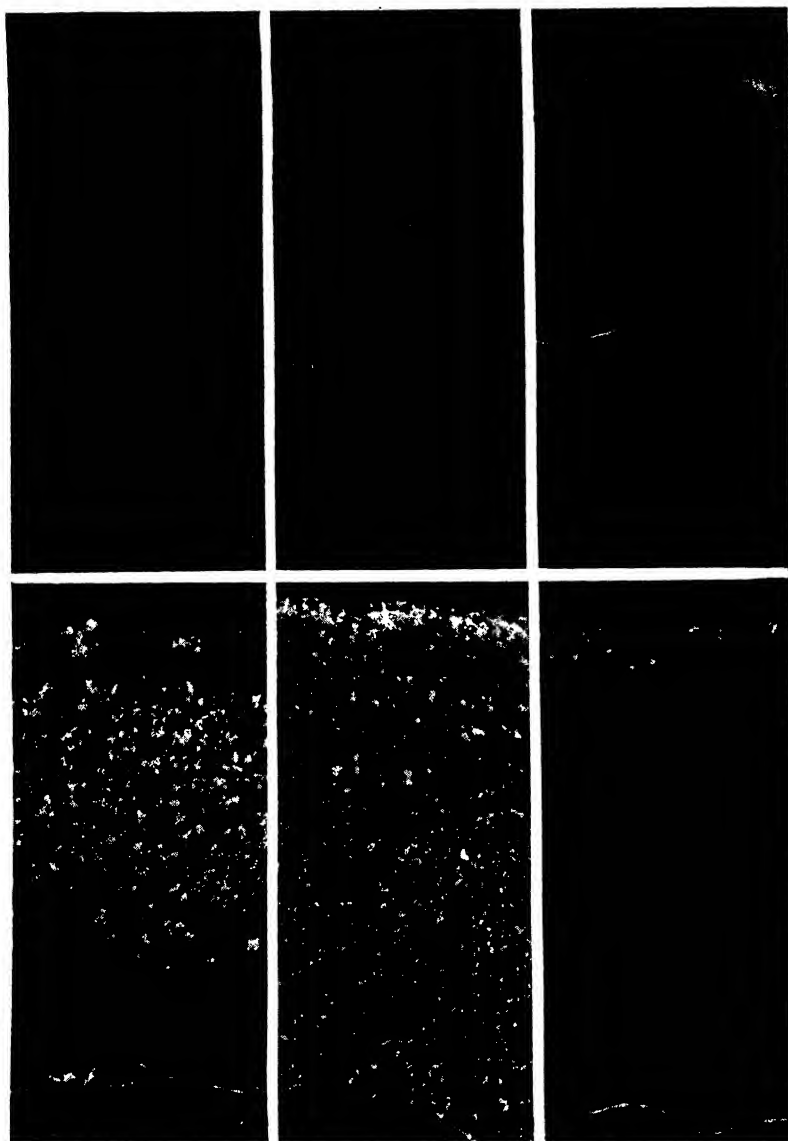
Depletion of lipids from the glomerulosa; fasciculata similar to that in FIGURE 14. FIGURES 14 and 15 illustrate the marked hypertrophy of the fasciculata under the influence of pituitary stimulation.

FIGURE 16. The normal rat adrenal cortex consists of a subcapsular glomerulosa, a transitional zone, a broad fasciculata, and an ill-defined juxtamedullary reticularis. The lipid droplets react strongly with the Schiff reagent in the glomerulosa and in the outer portion of the fasciculata.

FIGURE 17. Cortex of a rat hypophysectomized for 56 days. A marked atrophy of the inner zones has occurred, while the glomerulosa has broadened. Schiff-positive material has all but disappeared from the inner zones, but persists in high concentration in the zona glomerulosa. The maintenance of the glomerulosa suggests that this zone is independent of the pituitary.

FIGURE 18. Cortex of a rat hypophysectomized for 56 days and injected for the final 28 days with 2 mg. desoxycorticosterone acetate daily. The injections have caused further atrophy of the cortex and a complete disappearance of Schiff-positive lipid from the glomerulosa. The disappearance of lipids from the glomerulosa suggests that this zone has undergone disuse atrophy and, therefore, probably secretes desoxy-corticosteroids.





FIGURES 19-24

Adrenal cortices of rats. The border between the cortex and medulla has been drawn in. FIGURES 19-21 illustrate the autofluorescence of the adrenal cortex emitted under ultraviolet light. Autofluorescence characterizes the steroid hormones of the adrenal cortex. From DEANE & GREEP.<sup>18</sup> X 125. FIGURES 22-24 illustrate the distribution and quantity of birefringent lipids in the cortex, birefringence being a property of steroids. From DEANE & SHAW.<sup>19</sup> X 125.

FIGURE 19. The adrenal cortex of a normal rat. A yellowish- or greenish-white autofluorescence characterizes the lipid droplets of the glomerulosa and fasciculata. The transitional zone and the reticularis do not fluoresce.

FIGURE 20. The adrenal cortex of a rat hypophysectomized for 28 days. The marked atrophy of the gland is accounted for by the shrinkage of the inner zones. Only a trace of autofluorescence remains at the juxta-medullary border, while there is an augmented amount in the tissue derived from the glomerulosa. These observations suggest that the fasciculata, but not the glomerulosa, is under pituitary control.

FIGURE 21. The adrenal cortex of a rat hypophysectomized for 136 days. No further atrophy of the cortex

*Liebermann-Burchardl reaction.* This procedure, involving treatment with a mixture of concentrated sulfuric and glacial acetic acids, produces colored reaction-products with various unsaturated polycyclic compounds.<sup>24b</sup> Unsaturated steroids are prominent among these reactive substances. An adaptation of this reaction for histological purposes, the Schultz test, has long been thought specific for cholesterol, but it now appears to be a general steroid reaction.<sup>5</sup> This reaction has been used successfully on the ovary,<sup>22, 24a</sup> but to our knowledge has not been applied to the adrenal.

*Birefringence.* When observed in polarized light, droplets containing ketosteroids appear bright on a dark background. This property, which is caused by the radial symmetry of the steroid molecules in liquid spherocrystals,<sup>43</sup> is an expression, therefore, of the molecular pattern assumed by this class of compounds. Bennett<sup>2</sup> adopted the further refinement of pre-treating frozen sections with digitonin, which forms insoluble, anisotropic esters with cholesterol<sup>41</sup> and other  $\beta$ -steroids, thus producing more birefringence.

An important aspect of the study of birefringence in adrenal sections is the consideration of particle size. Accumulating evidence suggests that fine particles characterize active secretion, whereas large particles indicate inactivity or storage.<sup>19, 21, 65</sup>

*Autofluorescence.* The biologically active ketosteroids, including those of the adrenal cortex, exhibit fluorescence.<sup>5, 50</sup> This property is a result of the excitation of electrons in the molecule by the absorption of light quanta. The lipid droplets of the glomerulosa and fasciculata in adrenal sections viewed microscopically with ultraviolet light emit a yellowish or greenish-white fluorescence<sup>16, 34</sup> (FIGURE 19).

*Solubility.* Steroids are soluble in cold acetone. This property is utilized negatively in the evaluation of the above tests, since any material which retains its reactivity following such extraction cannot be considered steroidal.

In summarizing this section, it should be said that, although ketosteroids react positively with all of these procedures, no one of the tests is specific for these compounds. Thus, convergent evidence obtained by the simultaneous use of several or all of these methods is a more trustworthy indication of the presence of ketosteroids than is the result of any single reaction. Furthermore, no single class of substances, other than ketosteroids, exhibits positive reactions with the entire battery. Positive results from this series of reac-

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has occurred, but the inner zones are now entirely depleted of autofluorescent material. The glomerulosa, on the other hand, is even broader and also contains more fluorescent lipid than at 28 days.

FIGURE 22. Normal adrenal cortex of an adult rat. Birefringent particles of varying size characterize the glomerulosa and a broad segment of the fasciculata. The results of Weaver and Nelson<sup>50</sup> indicate that coarse particles represent storage material and that fine particles represent hormone in the process of release.

FIGURE 23. Cortex of a rat that had experienced a reduction in food supply for approximately 2 weeks. This figure illustrates the "resistance" phase of Selye's adaptation syndrome,<sup>58</sup> with the whole cortex filled with birefringent material. Many fine particles may be seen.

FIGURE 24. Cortex of a rat fed a thiamine-deficient diet for 4 weeks. Like pantothenic acid deficiency, a thiamine deficiency induces a typical adaptation syndrome. Complete depletion of birefringent steroids from the fasciculata has occurred, although the glomerulosa retains some. The restriction of the changes in quantity of steroids during the adaptation syndrome principally to the zona fasciculata leads us to believe that this zone secretes the 11-oxy-corticosteroids, the secretion of which is particularly augmented in conditions of stress.

tions indicate the presence of lipid-soluble, carbonyl-containing molecules, the anisotropy and autofluorescence of which are similar to those of known ketosteroids. It seems unlikely that other possible reactants would occur superimposed in such a fashion as to give positive results by all these tests.

The experimental material which will be reviewed below adds further circumstantial evidence favoring the thesis that the reactive materials are ketosteroids, since, in physiological situations where the production of hormones rises or declines, the tests wax and wane in a parallel fashion.

#### *Experimental Modification of the Ketosteroid Content of the Adrenal Cortex*

*Hypophysectomy.* The combined morphological and cytochemical changes which occur in the adrenal cortex after removal of the pituitary are of extraordinary interest. The adrenal cortex was early found to undergo such severe atrophy that it seemed unlikely that any part of its function could be retained. Enigmatically, however, hypophysectomized animals survived, while totally adrenalectomized rats succumbed unless given supplementary sodium chloride.

In the adrenal cortex of the rat after hypophysectomy, the fasciculata and reticularis atrophy the most severely.<sup>16</sup> The cells are reduced in size and eventually in numbers, so that, after 2 months, the zona fasciculata can scarcely be said to exist and cannot be distinguished from the reticularis. The glomerulosa broadens as the fasciculata shrinks and may increase from about 6 cell layers (normal) to 14 to 18 cell layers (compare FIGURES 19, 20, and 21). This increase in depth of the glomerulosa is due to a rearrangement of the cells on the periphery of a shrinking sphere, as well as, probably, to the addition of new cells.

We have followed the redistribution and changes in the reactions of the lipid droplets in the cortex of rats at intervals over a period of 4 months after removal of the pituitary. Along with the preservation of sudanophilia in the widened glomerulosa, we find that ketosteroids are likewise retained, as is indicated by the presence of acetone-soluble, birefringent, autofluorescent (FIGURES 20 and 21), and Schiff-positive (FIGURE 17) material. The transitional zone becomes definitely wider. With the slow leaching-out of lipid droplets from the shrinking fasciculata, the Schiff-positive and birefringent material also diminishes, but the natural fluorescence is lost most rapidly (FIGURES 20 and 21). Thus the fasciculata gradually loses the principal histochemical reactions indicative of the ketosteroids.

That the glomerulosa is not a conspicuous target organ of the anterior lobe of the pituitary is supported by the following evidence: after hypophysectomy, the cells maintain a healthy appearance; the thickening of the glomerulosa indicates that this zone is attempting to maintain its preoperative volume; and the persistence of Schiff-positive, birefringent, and autofluorescent material at normal or more than normal levels for at least 4 months is presumptive evidence of a continuing formation of ketosteroids. Since an array of physiological data indicates the continued secretion of salt-conserving corticosteroids following hypophysectomy,<sup>37, 61</sup> these observations suggest

that the glomerulosa is the source of such hormones, and that it is independent of the pituitary. (See, however, conflicting evidence for the human.<sup>3a</sup>)

The zona fasciculata, on the other hand, appears to be completely dependent on the hypophysis. The activity of the pituitary is required both for maintenance of cell size and for the formation of secretions in this zone. Since the 11-oxy-steroids, which are concerned with gluconeogenesis<sup>38, 44</sup> and the release of antibodies,<sup>67</sup> are apparently not secreted following hypophysectomy, their origin may, therefore, be attributed to the fasciculata.

*Stress.* The rôle of the adrenal cortex in the adaptation of the body to noxious agents or to environmental situations which create an unusual demand for energy has been intensively explored by Selye.<sup>57, 58</sup> The mechanism by which the adrenal produces such adaptation still remains somewhat obscure. It is widely accepted, however, that a heightened activity of the adrenal cortex is involved. The evidence is two-fold: (1) injections of adrenal extracts or 11-oxy-steroids better enable an animal to survive a stress,<sup>38</sup> and (2) in stress, the adrenal undergoes morphological and cytochemical modifications which constitute presumptive evidence of a rise in secretory activity.

Our concern here is with the latter evidence. It is of major importance that the glomerulosa fails invariably to take any great part in these modifications. The fasciculata, on the other hand, is subject, not only to an increase in volume (FIGURES 14 and 15), but to extreme alterations in lipid content and distribution. The adrenal changes which characterize the three stages of the adaptation syndrome, as defined by Selye,<sup>58</sup> are as follows: The immediate reaction to an alarming stimulus is an hypertrophy of the fasciculata with accompanying lipid depletion (the "alarm reaction"). During the "resistance" phase the lipid reaccumulates in the fasciculata and may be present in supranormal amounts (FIGURE 23). In the "exhaustion" phase, the fasciculata undergoes maximum hypertrophy and lipid depletion becomes extreme (FIGURES 14 and 24).

Observations on the effects of stress on the adrenal cortex have generally been confined to the changes in distribution of osmiophilic or sudanophilic material.<sup>12, 13, 21, 24, 35, 54, 55, 68</sup> That these methods alone are inadequate means of determining adrenal secretory activity has only recently been clearly recognized. The studies of Popják,<sup>48</sup> and of Deane and associates indicate that a complete parallelism does not obtain between sudanophilia and the more specific ketosteroid reactions.

Popják<sup>48</sup> studied the first phase of the adaptation syndrome by examining the adrenals of rats during the 48 hours following a traumatic injury. He performed parallel chemical and cytochemical analyses. Frozen sections stained with sudan IV were compared with adjacent sections treated with phenylhydrazine and others examined with polarized light. At 24 hours after injury, the depletion of sudanophilic and birefringent lipids from the fasciculata was associated both with an increase in the intensity of the yellow phenylhydrazine reaction, and with a widening of the zone showing the reaction. Chemically, he found a reduction of cholesterol during the same period. Thus, during the "alarm reaction," with a reduction of total lipid

as demonstrated by sudanophilia, birefringence, and total steroid, there was a rise of free ketones as demonstrated by the phenylhydrazine reaction.

Studying the whole course of the adaptation syndrome in rats<sup>17, 19</sup> and dogs<sup>46</sup> subjected to several different nutritional inadequacies, Deane and co-workers have observed a rise in sudanophilia in the "resistance" phase, followed by a decrease in the "exhaustion" phase which begins at the juxta-medullary border and continues peripherally (FIGURE 14). During the early part of the resistance phase, there is a notable increase in birefringence in the fasciculata (especially of fine particles indicating secretion), which is maintained for some time after the fall in sudanophilia begins (FIGURE 23). At the time of exhaustion, birefringence also drops (FIGURE 24). In this study, birefringence seemed to serve as a more significant index of steroid content and secretion than sudanophilia. This is probably because sudanophilia characterizes both triglycerides and steroids, whereas birefringence probably signifies only the latter. Interestingly enough, Oleson and Bloor<sup>47</sup> found, chemically, a decline of triglycerides in rabbit adrenals while the steroids continued to rise, during the "resistance" phase.

It has been well established that, under conditions of stress, an hypertrophy of the cortex and an increase and subsequent depletion of its lipid are associated with increased cortical secretion. This is indicated by the fact that the corticosterones afford added protection against alarming agents and also by the appearance of cortin-like substances in the urine of patients experiencing physical stress.<sup>66</sup> The adrenal changes may be attributed to an increased secretion of adrenotropin. Using adrenotropin, Bergner and Deane,<sup>3a</sup> have simulated the "alarm reaction." In confirmation of Popják's observations, there was a decrease in the amount of sudanophilic and birefringence material. The reduction in birefringence was accomplished, principally, by the loss of the coarse particles, whereas the residual particles were of the fine variety. Accompanying these changes, there was an increase in intensity of the Schiff reaction and of autofluorescence. It would seem, therefore, that these changes are associated with the active release of hormone from the fasciculata. Sayers and associates,<sup>56</sup> Levin,<sup>42</sup> and others have demonstrated chemically a similar fall in adrenal steroids after injecting adrenotropin.

*Disuse Atrophy.* It is a common endocrinological observation that a gland ceases to function and becomes atrophic if the hormone which it produces is artificially provided. In no instance have experiments of this nature been more revealing nor yielded more definite information than those concerned with the adrenal glands.

*1. 11-Oxygenated corticosteroids.* It has been amply demonstrated that injections of adrenal cortical extract or of the 11-oxygenated corticosteroids bring about a reduction in the size of the adrenal cortex.<sup>39</sup> Examined histologically, such glands show an atrophy confined to the inner zones, and the glands bear a remarkable resemblance to those of hypophysectomized rats. Deane, Thorn, and associates<sup>20</sup> have made a cytochemical study of the adrenal cortex after injections of corticosterone. The distribution pattern of the sudanophilic material is altered in exactly the same manner as that produced by hypophysectomy: the individual sudanophilic droplets and

birefringent particles in the fasciculata become larger as the zone atrophies. In contrast to these changes in the fasciculata, the lipids of the glomerulosa are unaffected.

Ingle<sup>37</sup> and others have commented on the possibility that the shrinkage of the fasciculata may be another instance of compensatory or disuse atrophy. This interpretation is so thoroughly indicated by circumstantial evidence that we feel justified in accepting it. Although the mechanism involved has not been entirely proven, the likelihood is very great that the corticosterones operate by suppressing the production of adrenotropin.

2. *Desoxy-corticosteroids*. The adrenals of normal rats treated with desoxycorticosterone acetate show a very different response from that seen after injections of 11-oxy-steroids. Carnes *et al.*<sup>10</sup> and Sarason<sup>54</sup> have observed that there is a disappearance of lipid from the glomerulosa following the prolonged administration of desoxycorticosterone acetate. We have found, after 7 to 12 days' treatment with desoxycorticosterone acetate, that the glomerulosa is less intensely sudanophilic and its autofluorescence is much less conspicuous. By 28 days, there are no longer any sudanophilic droplets in the glomerulosa, nor could we find a trace of ketosteroid by any test.<sup>31</sup>

Similar injections were made into hypophysectomized rats. The treatment was started 28 days after the operation, when the lipids of the fasciculata were greatly depleted and the glomerulosa was considerably thickened and laden with sudanophilic, birefringent, autofluorescent, and Schiff-positive material. After 28 days of treatment, the glomerulosa was completely negative to all these tests. Since the fasciculata was already atrophic, this latter treatment produced a cortex that was unreactive throughout (FIGURE 18).

These results are complementary to those produced by injections of corticosterone, and we attribute them to a similar phenomenon, namely, disuse atrophy. However, the mechanisms involved cannot be similar. Desoxycorticosterone does not operate through the pituitary to shut off the stimulus to the glomerulosa, since the reaction occurs in hypophysectomized animals. The mechanism which stimulates and regulates the glomerulosa is unknown. We can speculate that the secretory activity of the glomerulosa may be regulated by the concentration of one or more of the electrolytes in the body fluids, much as that of the parathyroid glands appears to be regulated by the blood level of ionic calcium.<sup>30</sup>

Using larger doses of desoxycorticosterone than those in our experiments, many authors have reported severe atrophy of the fasciculata as well.<sup>10, 11, 59</sup> Following such treatment, the osmic acid preparations of Carnes and associates<sup>10</sup> do not show any change in the relative amount of lipid in this zone, but further cytochemical studies are needed on such material to determine whether hormone production is normal (cf.<sup>31</sup>).

#### *Ascorbic Acid*

The known abundance of vitamin C in the adrenal cortex and the recent studies concerning its cyclic rise and fall in relation to cortical activity make the cytochemical investigation of this material highly desirable. Ascorbic

acid is the only known biological substance having the capacity to reduce silver nitrate at low pH. Histochemical utilization of this property permits the precise histological localization of this important acid. The acid silver nitrate is variously applied: (1) by perfusion<sup>28</sup>; (2) to unfixed, frozen sections<sup>7</sup>; or (3) in alcohol, to a freshly excised block of tissue.<sup>7</sup> In our hands, the last named method has been most successful. Following immersion in the silver nitrate-alcohol, the block is treated with acidified sodium thiosulfate, and then imbedded in paraffin, sectioned, and counterstained lightly with a nuclear stain.<sup>18</sup> The only disadvantage of these histochemical methods is that vitamin C may be present in an oxidized form and, therefore, not exhibit its strong reducing power. Thus, a positive reaction is certainly significant, but a negative one need not be.<sup>28</sup>

A number of observations on the distribution of ascorbic acid in the adrenal cortex have been published. The papers of Bourne<sup>7</sup> and Leblond and co-workers<sup>26, 27, 28, 40</sup> are the most notable. In the normal adrenal cortex of a variety of species, a precipitate of silver is seen to occur in all of the cells, distributed as fine granules in the zona glomerulosa (FIGURE 2) and as coarse ones in the fasciculata and reticularis (FIGURE 3). It is generally reported that there is less ascorbic acid in the glomerulosa than in the rest of the cortex.<sup>26</sup> Moreover, granules of reduced silver may also occur in the sinusoids, a phenomenon which suggests the possible secretion of ascorbic acid.

The precise intracellular localization of ascorbic acid is difficult to ascertain, because it probably exists in solution within the cell and may migrate before fixation is completed. In our experience, the speed of immersion of the block in the reagent clearly affects the distribution. Uniform distribution characterizes immediate fixation, and clumping in the Golgi region occurs when fixation is more leisurely. These results suggest the possibility of post-mortem migration.<sup>18</sup> For this reason, the many studies describing localization of vitamin C on the mitochondria<sup>27</sup> or within the Golgi apparatus<sup>8, 27</sup> seem to us of little significance.

There are now several chemical studies showing a concomitant decline in ascorbic acid and cortical steroids in sundry conditions involving stress.<sup>29, 56</sup> A cytochemical study has likewise demonstrated parallel disappearance of lipids and ascorbic acid from the adrenal cortex of the guinea pig in scurvy.<sup>4</sup> Cytochemical studies of the adrenal in other experimental conditions are now greatly indicated (cf.<sup>18</sup>).

### *The History of the Cells of the Adrenal Cortex*

The hypothesis that the cells of the adrenal cortex arise from the capsule and migrate centripetally in the cortical fascicles to die at the medullary border is widely held.<sup>2, 35, 71</sup> Through restatement, this idea has become entrenched in endocrine literature and thought. Most writers state or imply that the cells undergo a single cycle of secretion as they migrate inward, and on this basis each zone of the cortex represents one stage of the secretory cycle. Bennett<sup>2</sup> utilized this concept as a basis for interpreting the distribution of ketosteroids in the cortex in a histophysiological manner.

Actually, this concept of cell migration, which for brevity we will call the "escalator" theory, is founded on two assumptions, neither of which can be regarded as proven: (1) that mitosis occurs solely in the outer regions of the cortex; and (2) that cell death occurs almost entirely in the zone bordering the medulla.

*Cell Division.* In the adrenal cortex, cell division occurs predominantly in two localities, namely, in the glomerulosa and in the outer region of the fasciculata. Zwemer and associates<sup>71</sup> have been inclined to consider only the former location in their enthusiasm for the "escalator" theory. Hoerr<sup>35</sup> and Nathanson and Brues,<sup>46</sup> studying stimulated adrenals, observed mitotic figures throughout the cortex, but essentially disregarded those in the deeper layers of the cortex because of their belief in a steady inward flow or migration of the cells. Recently, however, with the repeated observation of the occurrence of mitosis in all sections of the cortex, workers have gradually come to doubt the acceptability of the "escalator" theory.

Two helpful studies of the distribution of mitotic figures are available. Baxter<sup>1</sup> found the number of arrested figures per 10,000 cells at 16 hours after the injection of colchicine to be:

capsule	52
zona glomerulosa	116
zona fasciculata	157
zona reticularis	66

Since colchicine may itself serve as an alarming stimulus, the exact relative distribution of mitotic figures as given here may not be an index of the distribution in the normal cortex. Nevertheless, it indicates the capacity for cell division in all the zones of the cortex.

Vaccarezza,<sup>62</sup> studying cell division in normal adrenals and those subjected to direct lesions, concluded that there is local replacement of cells anywhere in the cortex whenever death occurs in isolated cells. If, on the other hand, there is an extensive lesion, mitoses may occur at some distance from it, with the result that the area of injury is filled in by centripetal proliferation. Recently we<sup>32</sup> have studied the regeneration of cortical tissue following severe enucleation of the adrenal. Mitoses occur throughout the regenerating mass, rather than being limited to the subcapsular region. However, the new tissue appears to be derived entirely from cells which once lay in the glomerulosa. Since the new gland is capable of secreting both "salt" and "sugar" hormones, it seems clear that transformation of glomerulosa-derived cells into fascicular cells can take place under these extreme circumstances. Suffice it to say that under normal circumstances the distribution of mitotic figures does not support the assumption of continuous migration of cells from the capsule to the medullary border.

*Cell Death.* The reticular zone has been universally described as an area of cell destruction and death.<sup>2, 35, 70</sup> The evidence favoring this belief is impressive, and we do not deny that much cell destruction occurs here. We only submit that an obsession with the concept of cell migration has too often led histologists to believe cells die only in this zone. Many of the signs of so-called abnormality may merely be a response to the relatively poor



quality of the blood supply here. A similar condition obtains at the center of the liver lobule, where the supply of oxygen has been shown to be low. Here the cells contain lightly staining, fragmenting mitochondria and a poorly defined Golgi apparatus, and, also, frequently experience fatty infiltration.<sup>15</sup> Perhaps, when the adrenal circulation speeds up in the alarm reaction and these cells receive blood with more oxygen and essential food-stuffs, they may "recover." Thus, Flexner and Grollman<sup>25</sup> doubted the degeneracy of this zone when they found that it responded to a stress, concomitantly with the inner fasciculata, by increasing in width.

Bennett<sup>2</sup> used supravital trypan blue to detect dying cells.<sup>14</sup> Although he found unquestionable evidence of cell death in the reticularis, the point of great moment, for the purpose of the present discussion, is the large number of dying cells which he found in the other zones, particularly among the spongiocytes.

*Studies Employing Intravital Trypan Blue.* From histological examination of the adrenal cortex of several different species, Zwemer and co-workers<sup>71</sup> concluded that cells were constantly being contributed to the glomerulosa from what they called indifferent fibroblast-like cells located in the adrenal capsule. Knowing that fibroblasts ingest a small amount of trypan blue, Salmon and Zwemer<sup>53</sup> studied the adrenals of rats receiving intravital injections in the hope of marking the capsular cells so that their subsequent history could be followed. The trypan blue was first taken up by macrophages in the capsule, but, after 3 days, began to appear in the "fibroblast-like" cells which they believed gave rise to parenchymal cells. These investigators described the inner border of the vitally stained zone as migrating progressively deeper and deeper into the cortex, until, at 20 to 30 days, the zone of staining had reached the reticularis. In the analysis of these results, no distinction appears to have been drawn between the glandular cells containing fine particles of trypan blue and dye-laden cells lining the sinusoids. Furthermore, neither the length of time the dye remained in the blood stream nor the migratory habits of phagocytic endothelial cells were taken into consideration. Nevertheless, this study has been looked upon generally as providing of cell migration.

A preliminary reinvestigation of this work<sup>9</sup> has brought out the fact that the location of the dye in the cortex bears no relation whatever to the time elapsed after injection. Recently, Baxter<sup>1</sup> has carried out an even more detailed study and has found not the slightest evidence for the centripetal migration of cortical cells. The dye was phagocytized by individual cells in the capsule, glomerulosa, and reticularis within 24 hours. At 4 to 5 days, the dye also appeared in the zona fasciculata, but only in macrophages. He emphasized that the concentration of the dye in the blood stream did not fall for 6 days following an injection. Weatherford has made similar observations and come to the same conclusions.<sup>64</sup>

*Tissue Culture Studies.* Williams<sup>69</sup> utilized a moist chamber in the rabbit's ear for observing the behavior of transplants of isolated fragments of capsule, of glomerulosa, and of fasciculata from adrenal cortex. The cells which arose from capsular fragments were typical fibroblasts. The glomerulosal grafts became well vascularized and showed mitoses and cyclic secretory

activity which could be followed due to the refractile property of the cytoplasmic droplets. The fasciculata grafts did not become vascularized nor exhibit mitosis and were slowly reduced in size. The observation of importance for our purpose, however, is that, in the course of 8 months, there was no observed instance of the transmutation of a glomerulosa cell into the fasciculata type, nor *vice versa*.

*Evidence from the Location of the Reticulum.* Whether intercellular connective tissue exists in the cortex is important from the standpoint of the migration of the parenchymal cells. Hoerr described few reticular fibers penetrating between the cells of the fascicles and used this as an argument for the easy motility of the individual cells.<sup>35</sup> The reticulum illustrated by Gruenwald and Konikov<sup>33</sup> shows, however, a profusion of fibers standing as barriers across the supposed paths of migration.

### Summary

Sufficient differences in the form and chromophilia of the mitochondria and of the Golgi apparatus exist in active and inactive cortical cells to justify the belief that a definite correlation obtains between the character of these organelles and the state of secretory activity of the cells.

Ascorbic acid occurs throughout the adrenal cortex. It appears as fine granules in the cells of the glomerulosa and as coarse and crowded granules in those of the fasciculata and reticularis.

Ketosteroids may be characterized in frozen sections of the adrenal cortex by their reactions with sudan dyes, phenylhydrazine, semicarbazide, the Schiff reagent, and ammoniacal silver nitrate; furthermore, they are birefringent, exhibit a greenish-white autofluorescence, and are acetone-soluble.

The lipid droplets in the glomerulosa and the outer part of the fasciculata appear to contain ketosteroids by the above tests. The droplets in the cells of the inner part of the fasciculata and of the reticularis are presumably composed principally of triglycerides, since they are sudanophilic but exhibit only variable traces of the other steroid reactions.

In the hypophysectomized rat, the lipids and ketosteroids of the zona fasciculata are slowly lost, the mitochondria become smaller, and the Golgi knot more compact. The glomerulosa retains its sudanophilic, Schiff-positive, birefringent, and autofluorescent lipid droplets and becomes thicker as the adrenal atrophies.

In the adaptation syndrome there are pronounced changes in the adrenal cortex, but it appears that the glomerulosa plays no part in this reaction. The fasciculata displays a series of prominent morphological and cytochemical changes. In severe and prolonged stress, the mitochondria of the zone become swollen, less chromophilic, and eventually vesiculated, and the Golgi net becomes even larger and more extended than normal. The ketosteroid content is initially decreased, increases to above normal amounts during the "resistance" phase, then declines, and disappears terminally. The fasciculata enlarges after injections of adrenotropin; its ketosteroid reactions initially decline and later are supranormally augmented, while those of the glomerulosa remain unchanged.

In normal and hypophysectomized rats injected with desoxycortico-

sterone acetate the lipid and ketosteroid content of the glomerulosa slowly declines to extinction. Treatment with this substance does not alter in any way the reactions of the fasciculata. Conversely, after the administration of adrenal cortical extract or of 11-oxy-corticosteroids, the ketosteroid droplets of the fasciculata disappear.

On the basis of the observations that (1) cell division and cell death occur throughout the cortex, (2) *intravital* trypan blue fails to indicate translocation of cells, and (3) grafts of the adrenal capsule, glomerulosa, and fasciculata fail to undergo cytomorphosis into the other cell types, it may be concluded that the cortical cells generally arise, secrete, and die in their respective zones.

Our data support the following over-all view of the structural and functional interrelationship of the adrenal cortex. The zona glomerulosa, a seemingly autonomous region, in the rat at least, secretes desoxy-corticosteroids, which regulate fluid and electrolyte balance; the fasciculata secretes 11-oxy-corticosteroids, which are concerned with gluconeogenesis, resistance to stress, and the physiological mechanism of antibody production. The latter zone appears to be controlled entirely by the anterior pituitary.

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# THE ADRENAL CORTEX AND ITS TUMORS\*

By GEORGE W. WOOLLEY

*Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

It is evident, from cases taken from the literature, that in man a remarkable association between conditions of the adrenal cortex and genital and sexual changes has been recognized for many years. Males have been much less often affected than females.

The findings have been varied, both as to the age at which the symptoms appear, and as to the abnormalities and changes in primary and secondary sex characters which occur.

Recently, a new experimental approach has been made to tumors of the adrenal cortex in laboratory animals. The present report concerns studies made with the laboratory mouse. Since mice have a great similarity to man in their bodily structures, mice can conveniently be used in large numbers to establish principles which in turn may be modified for use with man. The adrenal tumor problem, a problem which has proven difficult to solve in man, may be one of these.

*The Adrenal Cortex.* It is interesting to approach the problem of the adrenal cortex by a review of the adrenal glands from a phylogenetic standpoint. Tissues corresponding to the interrenal body or the cortex of the mammalian adrenal have not been demonstrated anatomically in animals below the cyclostoma (hags and lampreys). The homologue of the chromophil or medullary tissue has been demonstrated in a number of lower invertebrates. In the anamniota (amphibia, fishes, and cyclostomes), the compact union of chromophil and interrenal tissue has not occurred and these tissues are represented by a number of bodies. Amphibia are intermediate in that there is a union of interrenal and chromophil tissue into a single organ without penetration of the latter tissue to assume a true medullary position. The adrenals of the bird show an interlacement of the interrenal and medullary tissues. It is only in the mammals that we find the interrenal tissue as a cortex surrounding a medulla composed of chromophil tissue. In man and in higher vertebrates, the adrenal in cross section is seen to consist of two parts, a soft, brownish-red, central portion, the medulla, and a surrounding outer portion, the cortex.

*The Adrenal Cortex of the Mouse.* Similar to man, the adrenal glands of the mouse are compound organs derived by union of two originally separate types of tissue. Because of this topographical relationship to one another, these tissues are referred to as cortical and medullary.

Waring<sup>3</sup> has studied the development of the adrenal gland of the mouse. The earliest cortical anlagen found was in an embryo of 12 days. It was distinguished from the surrounding mesenchyme solely by the closer aggregation of its nuclei and its more densely crowded cytoplasmic granules.

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The nuclei were essentially like those of the surrounding mesenchyme, being slightly ovoid and distinctly vacuolated. The anlage of the gonad was well developed at this stage. The fact that there was no connection whatever at this stage between the adrenal anlage and any other organ makes it extremely probable that the anlage had arisen *in situ* by proliferation from the peritoneum. By the end of the 13th day this anlage, which was destined to form the cortex of the adrenal, was consolidated and separated from the general mesenchyme by a sheath.

On the 13th day of foetal life there was a definite sympathetic mass closely applied to the cortical anlage. From it, sympathochromaffin elements eventually migrate into the cortical anlage to form the medulla of the adult adrenal. This sympathetic mass is very quickly developed, for in sections of 12-day-embryos no trace of it was found, while, at 13 days, it was well established. By the day of birth, the sympathochromaffin elements were concentrated at the center of the gland and were beginning to metamorphose into medullary cells. The undifferentiated eosinophil cortical tissue now formed a layer immediately outside the medulla. The lighter eosinophil staining tissue began to show the first distinct signs of the arrangement characteristic of the adult glomerulosa and fasciculata zones. After birth, in both sexes, the juxta-medullary zone increases, eventually increasing more in the female than in the male. This is known as the X zone. The three zones of the adult cortex are distinguishable in both sexes by 25 days: the zona glomerulosa, zona fasciculata, and the zona reticularis. At 35 days (exact time depending on strain), degeneration of the X zone of the male is well established. In the female, the X zone is now at about its maximum development. Degeneration of the X zone in the female is usually complete when the animal is between 100 and 300 days of age, depending upon the strain observed. The X zone of the mouse is probably homologous with the boundary zone of man and of the cat. In the mouse, it has been demonstrated that the X zone increases in width by cell division and not by change in appearance of cells in the inner layers of the permanent cortex. Using the colchicine technique, we have observed that, in the young, adrenal mitoses were evenly distributed throughout the zona fasciculata and the zona glomerulosa. Lack of frequent mitoses at the capsule indicated the absence of cell migration in the normal cortex at this age.

In certain strains of mice, particularly following gonadectomy, the development of the adult adrenal cortex is abnormal. Using the technique of gonadectomy, it has been found that much can be learned about the development of these abnormalities (tumors) of the adrenal cortex.

*Nodular Hyperplasia of the Adrenal Cortex.* The changes in the adrenal cortex of strain *JAX dba*, which has been studied extensively<sup>1, 4, 5, 6</sup> undergoes pronounced modification following gonadectomy. In all of the gonadectomized strain *dba* animals killed after 3 to 4 months of age, the adrenals have shown characteristic changes which progressively lead to the formation of nodular hyperplasia. Most of the glands were studied in serial section. The earliest changes consisted of the appearance of groups of small, densely arranged cells immediately below the capsule. Eventually these spread

between cells of the zona glomerulosa, zona fasciculata, and hypertrophied cells soon to be described. These subcapsular cells are polyhedral in shape, having deeply staining, round nuclei and slightly basophilic cytoplasm. As they extended in between the cell columns of the zona fasciculata they became spindle-shaped and strikingly resembled cells of the ovarian stroma. As the subcapsular cells increased in number by mitoses, they were seen first to interrupt the normal arrangement of the cells of the zona fasciculata and later to invade and replace the cells of all three zones of the cortex. This invasion soon resulted in the formation of a wedge shaped abnormal area. After the apex of the wedge reached the medulla, the sinusoid circulation of this part was disturbed and large, blood-filled lakes were often found surrounding the abnormal areas.

Simultaneously with the appearance of the subcapsular cells, cells which had undergone hypertrophy were found. These have been termed type A and type B cells, respectively. Normally, cells of the zona glomerulosa were small, with relatively large compact nuclei and slightly basophilic cytoplasm. Their cell outlines were indistinct. After undergoing hypertrophy, the nuclei became large and vesicular. The cytoplasm increased in amount, accumulated lipoid, stained lighter, and the cell outlines were more distinct. Such cells were occasionally found in the process of cell division. Later, these cells formed cell nests which were surrounded by spindle-shaped cells.

By increase in size and number of cell components, the wedge-shaped areas changed into round nodules and often involved large parts of the cortex. The structure of such nodules resembled those lutein cells of the mouse ovaries which develop from atretic follicles. The cytoplasm of these cells often contained yellow pigment. Occasionally, the pigmented cells fused and formed large multinuclear giant cells.

In some cases, the enlarging abnormal nodules caused bulging of the surface. In other cases, the nodules projected toward the center of the gland and pushed the medulla into an eccentric position. In several glands, enlargements occurred in both directions. Still further growth resulted in the invasion and finally in the breaking-through of the capsule, after which, a mushroom like outgrowth occurred and resulted in the involvement of the surrounding adipose tissue. In extreme cases, large areas of surrounding tissues were involved. Since circulation was seriously disturbed, necroses, thrombosis, and calcification in the central area have been seen to occur. The same adrenal frequently contained several abnormal nodules which showed different phases of the changes described. Even in the most extreme involvement, some normal cortical and medullary tissue was always present. The advanced changes were visible in the gross, enlarging the gland and giving it a nodular outline.

The same nodular hyperplasia occurred in gonadectomized male and female mice, although at any age period the changes tended to be more extensive and more advanced in the females than in the males.

*Adrenal Cortical Carcinoma.* In another strain studied extensively following gonadectomy,<sup>7, 8, 10, 11, 12</sup> strain JAX *ce*, other interesting tumors, adrenal cortical carcinomas, have been found. Preliminary changes toward

these tumors are very similar to the early changes toward nodular hyperplasia described for strain *JAX dba* and will not be described here. Adrenal cortical carcinomas first appeared in 100 per cent of the ovariectomized females at 6 months of age and were present in 100 per cent of the females in succeeding age groups. None were observed in the intact females of this strain examined. The same tumors occurred with a high frequency in gonadectomized males and not in intact males. As with nodular hyperplasia, the tumors of the male tended to develop later, and with a lower frequency, than in females.

In general, the smaller carcinomas were dense, cellular nodules composed of atypical cells showing frequent mitotic figures. They were imbedded in the outer region of the cortex, in areas already disorganized with changes toward nodular hyperplasia. Expansion into the medulla was evident in all cases as the nodules enlarged. Eventually the medulla and the remaining non-carcinomatous cortical tissue was spread out more or less as a sheet a few cells thick, first over much of the tumor and later, because of the large size of the tumor, over but part of the outer surface. The growths were fairly rounded in their earlier stages, but, as they attained a size of 1.5 cm. or more in diameter, protuberances often developed. The rounded contour was also modified by the adjacent kidney. Not only did the kidney modify the tumor, but the tumor often modified the shape of the kidney. That is, in some instances, the kidney was flattened on its anterior surface; in others, partially covered by the tumor. Invasion of the kidney was not noted, despite this close spatial relationship. Invasion of the liver and metastases to the lung have been observed.

The tumors were made up predominately of two types of tissue. Type I had large polyhedral cells with large oval nuclei containing chromatin granules of medium size. These cells were diffusely arranged. This tissue was not encapsulated, although the surrounding cell columns and sinusoids, which were compressed by the expanding growth at some places, gave this impression. Type II had small cells, cuboidal in shape, and arranged in rows. The cells had large, dark-staining, oval nuclei with evenly distributed coarse chromatin granules. A slight amount of slightly basophilic cytoplasm surrounded each nucleus (hematoxylin and eosin stain). The cells resembled the follicular cells of young ovarian follicles.

*Accessory Reproductive Organs.* The strain differences are further emphasized by observations made on accessory reproductive organs<sup>9, 10, 11, 12</sup> in the gonadectomized mice.

In strain C57 black, for example, little stimulation of the accessory reproductive organs occurs in the gonadectomized mice in later life. In strain *dba*, the stimulation is pronounced and is as if an estrogen was present. In strain *ce*, the stimulation is as if both estrogens and androgens were present: in some cases predominately like estrogen stimulation and in others predominately like androgen stimulation. In the case of strain *ce*, the stimulation is reproduced when the tumor is transplanted. Since transplantation data is for the most part unpublished, some of the observations will be presented here in considerable detail.



*Tumor Transplantation.* Within the series of gonadectomized *ce* strain mice with primary adrenal tumors, there were a number of variations in the nature of the accessory reproductive organ development. The present report concerns transplantation studies with 6 adrenal tumors from several of these different types of mice. The results indicate the necessity of evaluating adrenal cortical carcinomas individually, even in an inbred strain such as *ce*. They also indicate that considerable dependability exists for these tumors following transplantation.

All mice in this study were *ce* strain mice. Castration and ovariectomy was performed within 1 to 3 days after birth. Intact females were all virgin mice. The scale for tumor-size classification is the same as that previously used: microscopic to 0.5 cm. in diameter (small); 0.5 cm. to 1.5 cm. in diameter (medium); greater than 1.5 cm. in diameter (large). Since data on the primary tumors and their hosts have in some instances been presented in earlier reports, animal numbers and reference numbers are included for comparison of transplant with primary tumors. Tumor transplantation was accomplished by first cutting the tumor into small pieces with scissors on a piece of sterile glass. A few pieces of the tumor were then transplanted subcutaneously with the aid of a 13 gauge trocar. Both macroscopic and microscopic observations were made on the transplanted tumors and on many organs of the host mice. For histological study, tissues were fixed in a mixture of alcohol, formalin, and acetic acid,<sup>8</sup> embedded in paraffin, sectioned at 8 $\mu$ , and stained with hematoxylin and eosin.

1. The successful transplantation of one strain *ce* adrenal cortical tumor has been reported.<sup>13</sup> It was transplanted into four classes of animals: (1) gonadectomized females; (2) gonadectomized males; (3) intact females; and (4) intact males. The primary tumor originated in a gonadectomized female 17 months of age. The original tumor and the transplant tumors were associated with an influence which was interpreted as predominately androgenic on the basis of accessory reproductive organ observations.

2. A second adrenal cortical tumor originated in a gonadectomized male which was autopsied at 16 months of age. The primary adrenal tumor was of large size and the histological appearance was that of a typical *ce* strain adrenal cortical carcinoma. This tumor was of particular interest for transplantation because the original host had extensive squamous metaplasia of the prostate epithelium, indicating the presence of estrogenic hormones. Not all gonadectomized male mice with adrenal tumors had this metaplasia. The submaxillary gland was of male type, indicating the presence of androgenic hormones. The mammary glands were not uniformly developed from gland to gland; the most extensive were nearly as well developed as those of a *ce* strain virgin female one year of age. No end buds or alveoli were present.

The tumor was transplanted subcutaneously into a gonadectomized female when the host mouse was 37 days of age. This was the first transplant of this tumor. Autopsy was performed at 209 days, that is, after the transplant tumor had been present for 172 days. The tumor was then of large size. At autopsy the submaxillary gland was of male type. The clitoridean

glands were  $\frac{1}{4}$  the size of preputial glands in intact males of similar age. The vaginal epithelium was 4 to 5 cell layers thick. The outer layers stained lightly and were infiltrated with leucocytes. Leucocytes were also present in the lumen. The uterus was large in diameter, similar to a virgin female. There were well-developed uterine glands. The epithelial cells lining the lumen were low columnar and closely packed. The nuclei were oval and at the base of the cells. The mammary glands were similar to those of a virgin *ce* strain female of the same age. No nodules, so-called precancerous lesions, were present in the mammary glands of this mouse or any other mouse included in this report. The adrenal glands were without primary tumors. Foci of type A cells were present at the outer edge of the cortex. The cytoplasm of the cortical cells immediately below the type A cells stained lightly. Type B cells were not observed. On the basis of previous observations, extensive areas of type B cells, as well as small primary adrenal cortical tumors, would have been expected in an ovariectomized *ce* strain mouse of this age. Observations on the accessory sex organs are not incompatible with the interpretation that the tumor transplant produced internal secretions very similar to those produced by the original tumor.

The tumor was also transplanted subcutaneously into a gonadectomized male *via* the above ovariectomized female. The host mouse was 37 days of age at transplantation. Autopsy was performed when the mouse was 294 days of age. The tumor was of medium size at autopsy. The seminal vesicles were small and secretions were not present. There was extensive squamous metaplasia of the prostatic epithelium, as in the mouse with the primary tumor. The lumen of the prostate contained cornified epithelial cells and leucocytes. The submaxillary gland was intermediate between male type and female type, closer to female type than to male type considering the age of the animal. The mammary glands were not uniformly developed from gland to gland. However, all glands except L4 (numbering cephalad to caudad) were developed much more than those of an intact male, and were of medium length and narrow when compared to a strain *ce* virgin female of similar age. There were many small duct branches. No end buds or alveoli were present. One adrenal gland was without tumor. Type A and a few type B cells were present. The other adrenal possessed a tumor of the cortex which, at greatest diameter, occupied an area of about  $\frac{1}{3}$  the total width of the gland. Although no record of rate of growth of the tumor transplant was made, the fact that it was only of medium size after 257 days indicates that the growth had been slow. It might be interpreted that the small size of the transplant for several months permitted the occurrence of type B cells and of a primary adrenal cortical carcinoma. Internal secretions of a sex hormone nature in the primary tumor seemed to be similar to that in both transplant tumors, in that in each instance, there seemed to be a pronounced estrogenic effect.

3. Adrenal tumor P2528 originated in a gonadectomized male.<sup>12</sup> The mouse was autopsied at 21 months of age, at which time there were two adrenal tumors, the smaller on the right side. All transplants were of the tumor on the right side. The seminal vesicles were enlarged and secretion

copious. The prostates were well developed. Alveolar development of the mammary gland was extensive. The alveoli contained secretions. The submaxillary gland was male type. The anterior lobe of the pituitary was greatly enlarged.

Tumor P2528 was transplanted subcutaneously into a gonadectomized male when the host mouse was 34 days of age. Autopsy was performed when the animal was 327 days of age. The transplant tumor was large at this time (25 mm. in diameter). Grossly, it was observed that the seminal vesicles were  $\frac{2}{3}$  normal size. The prostates were well enlarged. The adrenal glands appeared to be normal. The submaxillary gland was large and light in color, similar to that of an intact male mouse. The preputial glands were  $\frac{1}{3}$  normal size. The pituitary was normal. The mammary glands were rudimentary. The histological appearance of the tumor transplant was the same as the original tumor. The microscopic appearance of the submaxillary gland, seminal vesicles, prostates, and preputial glands was similar to that in intact strain *ce* males. Small foci of type A cells were present at the outer edge of the cortex in each adrenal. No primary adrenal tumors were present.

Adrenal tumor P2528 was also transplanted subcutaneously into a gonadectomized female when the host mouse was 34 days of age. Autopsy was performed when the animal was 326 days of age. The transplant tumor was of large size. The uterus was medium large, but not well developed. The lumen was not extensively branched. The epithelial lining of the vagina was two cell layers thick. The clitoridean glands were almost the size of the preputial glands of an intact male. The submaxillary gland was male type. The tubular development was greater than is normally seen in *ce* strain male mice and could be identified in the unsectioned gland much as the tubules of a testis can often be seen through the tunica albuginea. The mammary glands were of medium length (compared to a virgin female), narrow, and with a few short branches. There were no end buds or alveoli. Type B cells were not present in the adrenal glands. No primary adrenal tumors were present.

4. Adrenal tumor P2511 originated in a gonadectomized female autopsied at 18 months of age.<sup>10</sup> At autopsy the mouse had two adrenal tumors. Parts of the larger (left) tumor were transplanted. In the original mouse, the submaxillary glands were male type. The uterus was large in diameter, with branched lumen and cystic uterine glands. The mammary glands were moderately developed; a few alveoli were present. The clitoris was unusually large in this female.

Adrenal tumor P2511 was transplanted subcutaneously in a gonadectomized female when the host mouse was 38 days of age. The tumor grew slowly, not being noticeable two months after transplantation. The mouse was autopsied at 376 days of age, at which time the transplant tumor was of medium size. The vaginal epithelium of the host was two cell layers thick, as in an immature mouse. The uterus was medium size in diameter. The submaxillary gland was male type. The clitoridean glands were about  $\frac{1}{2}$  as large as the preputial glands of a male. The clitoris was not unusually

enlarged. The mammary glands were of moderate length, narrow, and with many small branches. No end buds or alveoli were present. The right adrenal had a tumor of the cortex 2 mm. in diameter. The left adrenal had type A and type B cells but no adrenal tumor.

Adrenal tumor P2511 was also transplanted subcutaneously in a gonadectomized male when the host mouse was 38 days of age. At 373 days of age autopsy was performed. The transplant tumor was large at this time. At autopsy, the seminal vesicles were  $\frac{3}{4}$  the size of those in an intact male of similar age. The preputial glands were  $\frac{2}{3}$  normal size. The submaxillary gland was male type. The mammary glands were rudimentary. Type A and type B cells were present in each adrenal. No primary adrenal tumors were found.

5. Adrenal tumor P2214 originated in a gonadectomized female mouse.<sup>8, 9</sup> When the mouse was autopsied at 12 months of age, there was a medium-sized adrenal tumor on the left side. The right adrenal was without tumor. The epithelial layer lining the vagina was 12 to 14 cell layers thick. In addition, there was a cornified layer. The uterus was medium in diameter. The epithelial cells lining the lumen were tall columnar. Although the submaxillary gland was male type, its development in this direction was not pronounced. The mammary gland ducts were long and narrow. No end buds or alveoli were present.

Adrenal tumor P2214 was transplanted subcutaneously into an intact male P2565, when the mouse was 79 days of age. Autopsy was performed at 443 days, when the transplant tumor was of medium size. Grossly, it was observed that the seminal vesicles were slightly smaller than expected in a *ce* strain male of this age. Histologically, the seminal vesicles, prostates, and testes were normal. The mammary glands were rudimentary. The submaxillary gland was male type. No primary tumors or foci of type B cells were present in the adrenal glands.

Adrenal tumor P2214 was transplanted subcutaneously in gonadectomized female P2694 via P2565, when the mouse was 42 days of age. The mouse was autopsied when 22 months of age, when the transplant tumor was of large size.

At autopsy the uterus was medium large in diameter. The lumen was well branched. The epithelial cells lining the lumen were low columnar. The vaginal epithelium was two cell layers thick. The submaxillary gland was male type. The mammary glands were similar to those of a *ce* strain virgin female.

6. Adrenal tumor P2182 originated in an ovariectomized female which was autopsied when 10 months of age.<sup>8, 9</sup> At this time, there were two primary adrenal cortical carcinomas forming a tumor mass medium in size.

In the original mouse, the submaxillary glands were intermediate between male and female in type. The vaginal epithelium was 11-12 cell layers thick with a *stratum corneum* in addition. The basal layer of cells were dark staining. There were many cornified cells and a few leucocytes in the lumen. The uterus was of medium size with well-developed uterine glands. Closely packed, tall columnar cells lined the lumen. The cyto-

plasm of these cells was turbid appearing. A few mitotic figures were present. The blood supply of the uterus was very good. The thyroid had many small follicles. The epithelial cells lining the follicles were cuboidal. The follicles were not all completely filled with secretion. The mammary gland ducts were long, and end buds were present, indicating rapid growth. No alveolar development was observed.

Adrenal tumor P2182 was transplanted into 29 intact *ce* strain mice over 4 transplant generations. It grew successfully in 11 of 20 male mice and in none of 9 female mice. Tumor tissue and other tissue was saved for histological examination from male mice in the first 4 transplant generations. At first, the transplant was almost entirely of type II tumor tissue, a type which had previously been observed to grow with difficulty in competition with type I. In the first and second generation transplants, areas with less cell polarity and with lighter staining cells appeared within the tumor and seemed to be developing into a prominent part of the tumor. In later generations, this tissue was the extensive type.

The testes and accessory reproductive organs of the male mice with tumor transplants were not greatly modified by the tumor transplant. The adrenal glands had wide bands or areas of lipochrome cells between the medulla and cortex.

*Pituitary Relationships.* There is some evidence that the pituitary has a relationship to these adrenal cortical tumors. Although the problem needs much more study, we have observed that pituitary tumors (anterior lobe abnormalities) may occur in association with the adrenal cortical hyperplasias and carcinomas. These are being investigated in studies now in progress. These pituitary abnormalities did not occur in the control animals. Some evidence has been presented that these pituitary changes are correlated with extensive alveolar development of the mammary glands.<sup>10</sup> Furthermore, in one transplanted adrenal tumor (P2528), where there was an association between pituitary abnormality, adrenal cortical carcinoma, and good alveolar development of the mammary glands, it was observed that, in the mice with transplants of this adrenal tumor, the accessory reproductive organs were well stimulated but the mice did not have either abnormal pituitaries or extensive alveolar development in the mammary glands.

*Prevention of Tumors.* The prevention of nodular hyperplasia of the adrenal cortex and of adrenal cortical carcinoma is being investigated. The most extensive data comes from subcutaneous implantation of pellets of diethylstilbestrol in gonadectomized strain *ce* male and female mice.<sup>14</sup> In this study, fusion pellets of 25 per cent diethylstilbestrol in cholesterol, average weight 4.8 mg., were used. These were introduced into the subcutaneous tissue of the right axilla when the mice were approximately 7 weeks of age. Only one pellet was used at this age in each mouse and none were implanted later in life. Experimental mice were prepared by removing the gonads of strain *ce* mice immediately after birth. It will be recalled that adrenal cortical carcinomas had previously been observed in 100 per cent of gonadectomized female mice of this strain 6 months of age and older. In gonadecto-

mized male mice, these tumors had occurred starting at 7 months. Diethylstilbestrol-treated mice of both sexes observed up to 14 months of age did not have these tumors. Although data are not available for protection at extremely advanced ages, the results are encouraging for continued protection, since nodular hyperplasia of the adrenal cortex, which preceded the occurrence of carcinomas in previous experiments, was also prevented by the use of diethylstilbestrol pellets. Further study needs to be made to find protective agents for these tumors which will not at the same time cause other serious body disturbances. In addition to undesirable histological changes in the accessory reproductive organs, pituitary tumors of the type caused by estrogenic hormones were observed in the mice as early as 7 months of age.

*Genetic and Endocrine Factors.* There are important genetic factors back of the occurrence of adrenal cortical tumors. This is indicated by pronounced strain differences in response to gonadectomy. The adrenal cortex of strain *JAX C57 brown* or *JAX C57 black*, for example, undergoes only slight change in size or structure following gonadectomy. Strain *JAX dba* develops nodular hyperplasia of the adrenal cortex and strain *JAX ce*, adrenal cortical carcinoma. Hybridization experiments now in progress also point to the importance of the genetic factors.

Endocrine factors are also of great influence. In our series, adrenal cortical tumors of the types described in this report did not occur without gonadectomy. Following gonadectomy, certain endocrine preparations will prevent their occurrence in a genetically susceptible strain. It is known that there is an intimate relationship between the adrenal cortex and the pituitary. In these first experiments, the relationship is evidenced by occasional abnormalities of the anterior lobe of the pituitary in the experimental and not in the control series.

An hypothesis might be that there is: (a) an increased inactivation (utilization) of adrenal cortical hormone following gonadectomy; (b) the fall in cortical hormone level (or possibly only the fall in gonadal hormone level) removed an inhibitory influence on the adrenocorticotrophic activity of the anterior lobe of the pituitary; and (c) increased adrenocorticotrophic activity led to hypertrophy of the adrenal cortex. Cortical carcinoma occurrence of the type described is the result of certain genetic factors, together with a certain balance of the endocrine factors.

It is hoped that eventually the problem will be reduced to: how the adrenal cortical carcinoma originates from responsible factors.

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# URINARY EXCRETION OF ADRENAL CORTICAL STEROIDS

By ELEANOR H. VENNING AND J. S. L. BROWNE

*McGill University Clinic, Royal Victoria Hospital, Montreal, Canada*

It has become apparent in recent years, through the work of many investigators, that the adrenal cortex has a variety of functions. The substances so far derived from it may be roughly divided into three groups: (1) compounds which act mainly on electrolyte metabolism; (2) compounds which affect protein and carbohydrate metabolism, and (3) compounds which have properties of the sex hormones. Into the last group fall the estrogens, progesterone, and the androgens. The latter also affect protein metabolism.

Metabolites of these various adrenal hormones are excreted in the urine and an attempt has been made to correlate these urinary substances with their possible precursors in the gland or with definite types of adrenal function. In man, two groups of urinary steroids have been associated with adrenal metabolism, namely, the 17-ketosteroids and the glyco-genic corticoids.

The first group, the 17-ketosteroids, are characterized by the facts that they have a carbonyl group at C-17 and give a typical color reaction with alkaline dinitrobenzene. They have been associated with the adrenal, because they are found to be increased in cases of adrenal tumor and decreased in cases of hypofunction of the adrenal. In the male, the 17-ketosteroids are derived from both the adrenal and the testis, whereas, in the female, they are entirely of adrenal origin. The adrenal precursors of these substances have not yet been identified, but they are considered to be associated with the androgenic function of the adrenal, and to have an anabolic effect on body protein.

The glyco-genic corticoids differ from the 17-ketosteroids in that they possess the same biological activity as the corticosterones, *i.e.*, they are capable of prolonging the life of adrenalectomized animals and will cause an increase in liver glycogen when administered to fasting adrenalectomized animals. They are readily extractable from urine with chloroform or ethylene dichloride, are strongly reducing substances, and are heat labile. They are associated with that group of adrenal substances which are concerned with carbohydrate metabolism.

The urinary corticoids have been assayed by the method of Venning, Kasmin, and Bell.<sup>1</sup> It is a bioassay based upon the ability of these hormones to cause a deposition of glycogen in the livers of adrenalectomized fasted mice. Briefly, the assay is carried out in the following manner: The urinary extract is prepared by acidifying a 48-hour specimen of urine to pH 1 and extracting it with ethylene dichloride or chloroform. After distilling off the solvent, the residue is taken up in chloroform and the solution is extracted with N/10 alkali and water. The chloroform is evaporated off and the residue obtained is dissolved in a small amount of alcohol and is finally made up into an extract containing 10 per cent alcohol



and 5 per cent glucose. This extract is injected into mice which have been adrenalectomized 4 days previously and fasted for 16 hours prior to the assay. The extract is administered in 7 divided doses over a period of  $5\frac{1}{2}$  hours, and, 1 hour later, the livers are removed and are analysed for glycogen. The amount of glycogen deposited is compared with amounts laid down by 17-hydroxy 11-dehydrocorticosterone. One glycogenic unit is equivalent to the activity of 1 microgram of this compound. The administration of small amounts of glucose greatly sensitized the method, so that adrenal cortical steroids in the order of 8-10 micrograms can be detected. Thus, the measurement of the urinary corticoids offers a means of assessing a type of adrenal function different from that associated with the excretion of 17-ketosteroids. In the following study, the variations in the excretion of these two groups of adrenal metabolites have been investigated in normal individuals and in patients with various endocrine disorders. The effect of stress and trauma, such as severe muscular exercise, infections, burns, and fractures, has also been studied.

*Excretion in the Normal Individual* (FIGURE 1).<sup>2</sup> In 14 normal adult women, aged from 21 to 48 years, urinary corticoid activity ranged from 25 to 65 glycogenic units per 24 hours, with an average value of 41 units. In 14 normal adult men, between the ages of 20 to 51 years, the corticoids varied from 40 to 85 glycogenic units per 24 hours, with an average of 60 units. In the females, the 17-ketosteroids ranged from 6-18 mg./24 hours with an average value of 11.6 mg., while, in the males, they ranged from 10-25 mg./24 hours, with an average value of 16.6 mg. In old age, the values for both these fractions are decreased.

The urine from eight new-born male infants was collected during the first four days of life and an amount of urine equivalent to a 12-hour specimen was injected per mouse. In four of these cases, no activity could be detected; in four, amounts in the order of 10-15 units were found. By the ages of  $2\frac{1}{2}$  and 3 years, 35 to 40 glycogenic units were present in the urine and at  $5\frac{1}{2}$  years, adult male values were present. The 17-ketosteroids were low in the younger children, increased in value with advancing age, and reached adult values later than the corticoids.

Some variation is seen in the excretion of these substances when followed from day to day in the same individual, both in males and females, but values remain within the normal range established by single assays in a number of different individuals. Similar variations are observed in the excretion of 17-ketosteroids. The values for 17-ketosteroids and for glycogenic corticoids do not necessarily parallel each other even in the adults. No correlation could be found with the rhythmic excretion of the sex hormones in the menstrual cycle.

*Excretion in Endocrine Disorders* (FIGURE 2).<sup>3</sup> Our studies were extended to examine the excretion of these two groups of compounds in various endocrine and other disorders:

(1) *Addison's Disease*: Four cases were studied, one male and three females, all showing typical symptoms and signs of this disease. In all four

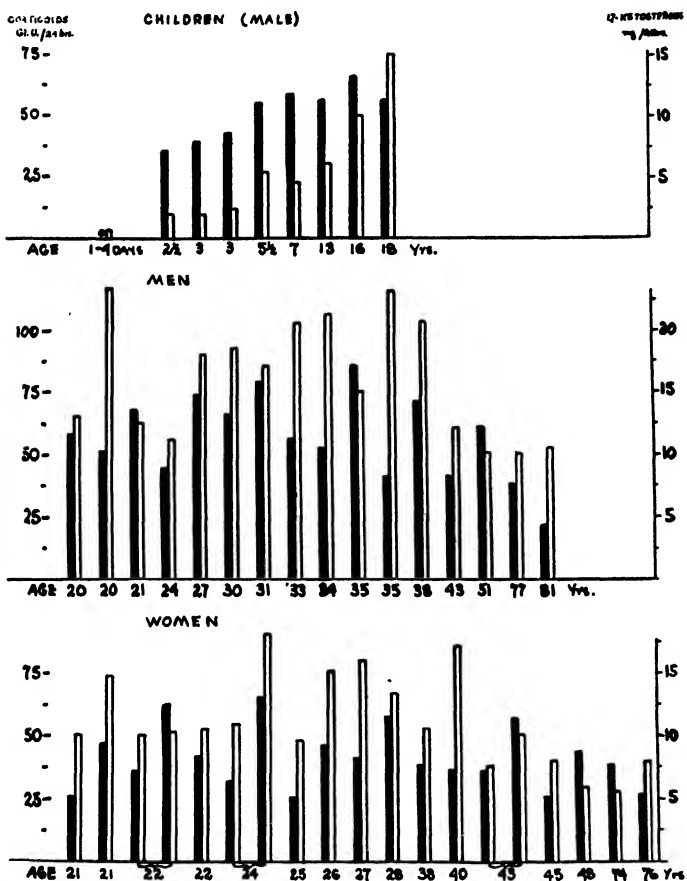


FIGURE 1. Excretion of glycogenic corticoids and 17-ketosteroids in a group of normal individuals. The black columns represent the daily excretion of corticoids in terms of glycogenic units; the white columns, mg. of 17-ketosteroids.

cases, the 17-ketosteroids were low. In three of the cases, less than the amount of glycogenic corticoid activity detectable by the method was present. In the fourth case, however, amounts varied from below normal to low normal on various occasions, suggesting the possibility that one type of function of the adrenal may decrease more rapidly than another, or independently of the other functions.

(2) *Panhypopituitarism*: Five cases were studied, three males and two females. All showed many signs of pituitary hypofunction. Both the 17-ketosteroids and the glycogenic corticoids were very low. It is of interest that these individuals did not show marked evidence of electrolyte disturbances in spite of the similarity of their glycogenic corticoid excretion to that found in Addison's disease. This may be further evidence for the possibility of dissociation between two types of adrenal hypofunction.

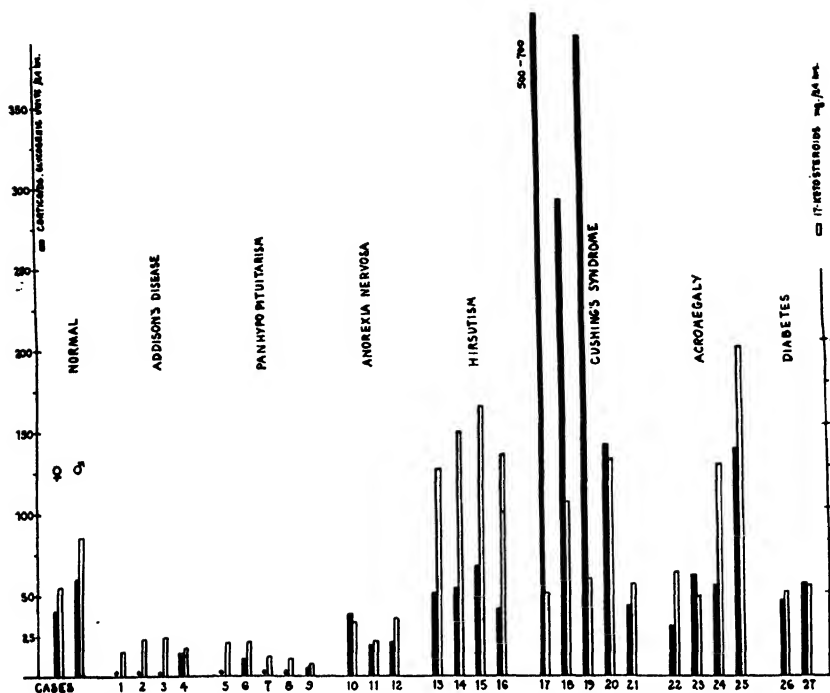


FIGURE 2. Excretion of glycogenic corticoids and 17-ketosteroids in various endocrine disorders.

(3) *Anorexia Nervosa*: Three cases which are typical as regards history and physical findings were studied. In these cases, the pituitary hypofunction has been regarded as occurring as a consequence of the malnutrition. The 17-ketosteroids were low, but in general not so low as those found in the cases of panhypopituitarism, described in the previous section. Glycogenic corticoids were present in all three cases, but in amounts lower than normal.

(4) *Hirsutism (Simple)*: In four cases of simple hirsutism, the glycogenic corticoids were normal, while the 17-ketosteroids were definitely raised, ranging from 25 to 35 mg./24 hrs. None of these patients showed any other signs of virilism except the increase in body and facial hair. Only in Case 16 were the adrenals explored and, in this case, the right adrenal was slightly enlarged.

(5) *Cushing's Syndrome*: In all the cases of active Cushing's syndrome studied, the values for glycogenic corticoids were markedly raised, reaching values of 700 units in two cases. The 17-ketosteroids were either normal or slightly elevated. In certain cases where the process had been arrested or had become inactive, normal values were found, even though the patient still showed clinically the same evidences of Cushing's syndrome (Case 21). The degree of impairment of the glucose tolerance curve did not always parallel the increase in corticoid excretion.

(6) *Acromegaly*: In one untreated case of acromegaly of recent origin,

elevated glycogenic corticoids and 17-ketosteroids were found. In three other cases of long standing duration, in whom the pituitary had been irradiated, normal values for both corticoids and 17-ketosteroids were present.

(7) *Diabetes*: Several cases of diabetes have been studied and all have shown normal values. Three cases which were insulin-resistant did not have elevated glycogenic corticoids.

In general, it may be said that the determination of urinary glycogenic corticoids, in addition to the 17-ketosteroids, is of assistance in elucidating various types of cases whose signs and symptoms suggest abnormality of adrenal function. The two determinations frequently do not parallel each other, thus suggesting that the two types of adrenal function associated with these metabolites may be independent of one another.

#### *Other Factors Influencing the Excretion of Glycogenic Corticoids.*

(1) *Muscular Exercise*: It was found that strenuous muscular exercise caused a marked increase in output of these adrenal substances. On two separate occasions, pooled urine was obtained from a group of 14 healthy army recruits before and after a 4 mile route march. The glycogenic corticoids were increased from 46 to 160 glycogenic units per liter in the first experiment, and from 60 to 210 units in the second one. The 17-ketosteroids were not determined.

(2) *Effect of Trauma*<sup>4</sup>: During the past three years, in collaboration with metabolic studies on protein metabolism, an extensive investigation has been carried out on the excretion of glycogenic corticoids following acute trauma, such as surgical operations, burns, fractures, wounds, and infections, in both the previously healthy individual and the chronically debilitated person. The results may be summed up very briefly as follows: When a healthy well-nourished individual is subjected to an acute trauma, there is an immediate and rapid increase in output of the glycogenic corticoids, reaching a maximum in most cases within 3 to 4 days. This elevated output is maintained for a varying period of time, depending to some extent upon the severity of the damage inflicted. In most cases, the corticoid excretion has returned to the normal level by the end of the third week, but, in several cases, the increased excretion of corticoids is maintained for a much longer period. In some of the cases studied, values around 400 units have been found at the peak of the excretion, although generally, the values lie between 200 and 300 units. There is usually an immediate rise in 17-ketosteroids, but this increase is not always maintained and the changing levels of 17-ketosteroids and corticoids do not necessarily parallel one another.

In FIGURE 3 is shown the rapid increase in excretion of glycogenic corticoids and 17-ketosteroids in a well-nourished individual following fractures of the humerus and femur. On the 12th day after injury, a bone plating to the left femur was done. This second trauma was followed by a secondary rise in corticoid excretion. By the 15th day, the corticoids were back to normal and remained at this level throughout convalescence.

In the chronically ill person suffering from malnutrition, the response of

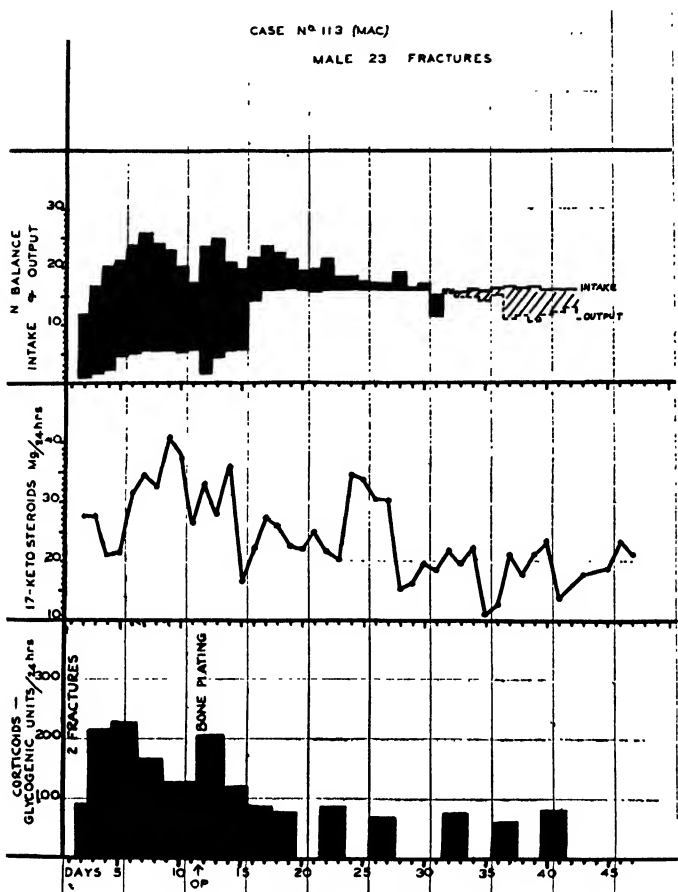


FIGURE 3. The excretion of glycogenic corticoids, 17-ketosteroids, and nitrogen balance in a well-nourished individual following fractures.

the adrenal to trauma is markedly different from that seen in the healthy individual. In a large percentage of the cases studied, the excretion of corticoids in these patients is above the normal limits. Following operation, there may be no increase in output of glycogenic corticoids, or else, the increase is maintained for only a few days, reaching normal levels again by the end of the first week.

*Excretion in Pregnancy.*<sup>5</sup> Earlier investigators had noted by direct observation that the adrenal gland undergoes definite hypertrophy during pregnancy. Nine cases in all were studied. In general, the curves obtained for the excretion of corticoids and 17-ketosteroids followed the same pattern, although some of the patients excreted larger amounts of metabolites than others. During the first trimester, all showed an increased excretion of corticoids. After this initial rise, the excretion returned to normal levels, then, between the 140th and the 160th days, began to rise again, reaching

values over 300 units in some cases. There was usually a falling-off in output before parturition. Within a few days after delivery, the urinary corticoids were back to normal level again. In FIGURE 4 are shown the

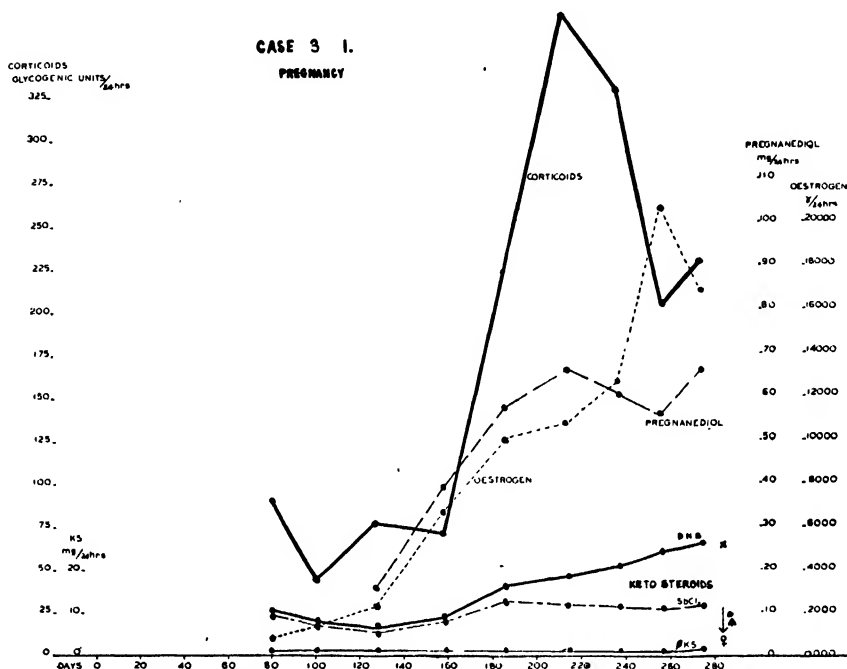


FIGURE 4. Excretion of glycogenic corticoids, ketosteroids, pregnanediol, and oestrogens in a case of normal pregnancy. The arrow denotes the time of delivery; X = corticoid excretion after delivery.

excretion rates of various hormones, including the corticoids, in a case of normal pregnancy.

The ketosteroids were followed by two colorimetric determinations: (a) color reaction with alkaline dinitrobenzene (this method includes both 17- and 20-ketosteroids); (b) color reaction with antimony trichloride (this method is more specific for 17-ketosteroids). It was found that with the reagent antimony trichloride no increase of 17-ketosteroids occurred, while with alkaline dinitrobenzene there was an increase in color index beginning between the 140th to 160th day. This increase in ketosteroids as measured by the Zimmerman reaction is attributed to the increased excretion of 20-ketosteroids.

### Summary

The excretion of urinary corticoids and 17-ketosteroids has been studied in a series of normal individuals and in endocrine disorders and other pathological cases.

These two groups of urinary metabolites represent different adrenal functions, and, from their excretion rates under various condition, it is suggested that these functions may vary independently of one another.

Strenuous exercise and acute trauma cause an increase in the output of the glycogenic corticoids. The well-nourished individual shows a more marked response than the malnourished one to damage.

In pregnancy, there is a rise in urinary glycogenic corticoids, but no parallel increase in 17-ketosteroids.

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# ADRENAL CORTEX FUNCTION IN STRESS

By GREGORY PINCUS\*

*The Worcester Foundation for Experimental Biology, the Memorial Foundation for Neuro-Endocrine Research, and the Department of Psychiatry of the Massachusetts General Hospital, Boston, Massachusetts*

*Introduction:* Animals and men respond to a great variety of damages and stresses with prompt secretion from the adrenal cortex.<sup>1-3</sup> We have found that this typical adrenal-cortex response to short, acute stress is deficient in psychotic men generally.<sup>3-6</sup>

The ordinary concept of the stress response is based on studies of the alarm reaction in which (a) the stress stimulus activated the anterior pituitary to secrete adrenocorticotrophic hormone and (b) the adrenal cortex, stimulated by the adrenocorticotrophin, released corticosteroid that (c) induces certain target organ activity involving such phenomena as lymphatic tissue breakdown, potassium release from muscle, glycogen deposition in liver, and so on. In this paper, we shall present data on men and animals which are pertinent to each of these three phases of the alarm reaction sequence. Our ultimate objective is to understand the significance of the disturbance of this mechanism in mental disease.

In this paper, we should like, first of all, to present what evidence we have concerning the degree of dependence of certain stress-response indices upon the adrenal cortex in animals and men. Secondly, we present data on the behavior of these indices in psychotic men under stress. Finally, we have data on psychoneurotic subjects to compare with those assembled for normal and for psychotic subjects.

*Indices of Adrenal Cortex Secretion.* We have previously reported data on two measures of adrenal cortex secretion in man: the 17-ketosteroid excretion and the number of circulating blood lymphocytes.<sup>3-6</sup> The latter can also be used as an index of the secretion of 11-oxygenated corticosteroids<sup>7,8</sup> in animals. In this paper we shall present data on other biochemical variables that reflect aspects of adrenal cortex function. These are: (a) the neutral reducing lipids of urine, which represent in large part the corticosteroids possessing a sugar-like (reducing) side chain<sup>9,10</sup>; (b) urinary uric acid, which represents the purine metabolite excreted in greatly increased amount after adrenal cortex hormone administration<sup>11</sup>; and (c) urinary potassium and sodium, which alter notably in amount after stress,<sup>1</sup> and the excretion of which is governed in large part by certain adrenal cortex steroids.<sup>12</sup>

Our objective in employing these various indices of adrenal cortex secretion is to see if different stresses affect any of them differentially in either normal or psychopathic subjects.

\* Research Professor of Physiology, Tufts Medical College, Boston, Mass.

These researches were conducted in collaboration with Drs. H. Hoagland, H. Freeman, J. Finesinger, A. Watkins, M. Brazier, E. Shantz, Messrs. F. Elmadjian, J. Carlo, D. Stone, and Mrs. L. Romanoff. Grants-in-aid were received from the National Research Council Committee on Endocrinology, the Baruch Committee on Physical Medicine, and G. D. Searle and Co. The Upjohn Company kindly supplied the adrenal cortex extract used.



*Glucose and the Alarm Reaction.* The lymphocytopenia following stress and the administration of 11-oxocorticosteroids may be rather faithfully simulated by the administration of glucose to both rats<sup>13</sup> and men.<sup>14</sup> FIGURE 1 presents data on normal and adrenalectomized rats subjected to one-

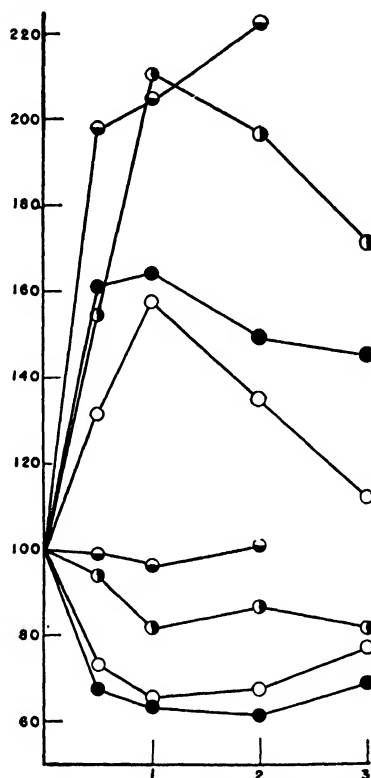


FIGURE 1. The mean percentage changes in blood sugar and lymphocytes following the administration of dextrose to rats. Abscissa: time in hours. Ordinate: per cent. ○ = normal rats, one dose test; ● = normal rats, two dose test; ◐ = adrenalectomized rats, one dose test; ◑ = adrenalectomized rats, two dose test. The blood sugar values are in the upper portion of the figure, denoting increases in all groups; the lymphocyte values are in the lower portion of the figure. (From *Endocrinology* 39, 1946)

dose and two-dose sugar tolerance tests. The fact that the adrenalectomized rats show no significant lymphocytopenia during the test, whereas the normal rats do, indicates that the blood lymphocyte changes are mediated by the adrenals. It is notable that there is a significant negative correlation between the lymphocyte and the blood sugar changes during the tolerance test in the normal rats, whereas no such correlation is found in the adrenalectomized animals (FIGURE 2).

Freeman and Elmadjian<sup>14</sup> have shown that a lymphocytopenia occurs in normal men in the two-dose (Exton-Rose) sugar tolerance test. Through the kindness of Drs. Fuller Albright and E. B. Astwood, we have been able to conduct Exton-Rose glucose tolerance tests with three Addisonian patients. In addition to the blood sugar and lymphocyte values, we have

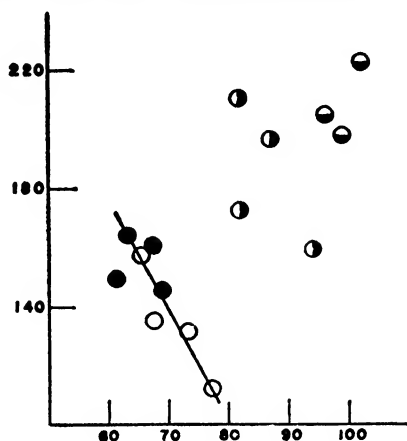


FIGURE 2. The mean percentage changes in blood sugar are plotted against the percentage changes in lymphocytes. Abscissa: lymphocyte percentages. Ordinate: blood sugar percentages. Symbols as in FIGURE 1. Note that the data for the normal rats indicate that rising blood sugar is accompanied by relative lymphocytopenia, and that this does not hold with the data for adrenalectomized rats. (From *Endocrinology* 39, 1946)

determined neutral urinary reducing lipid and urinary uric acid in these subjects. A comparison of the data obtained with them and on a group of five normal men examined at the same time is presented in FIGURE 3. The normal men exhibit the characteristic sugar tolerance curve with an accompanying blood lymphocyte decline during the first hour. The Addisonian patients show a distinctly abnormal tolerance curve with some decline in lymphocytes which is, however, proportionately much less than that observed in the normal subjects. The latter show a significant 17 per cent decline at the end of the hour's test, whereas the patients exhibit a 7 per cent drop which is not statistically significant. The normal subjects respond to the administration of sugar by excreting increased amounts of neutral reducing lipid, whereas the characteristic extremely low level of these substances in the patients' urines is scarcely affected. Although neither group show any significant urinary uric acid changes, the extremely low level of output in the Addison's cases is noteworthy.

The foregoing is taken to demonstrate that, in both rats and men, the administration of glucose leads to the increased production by the adrenal glands of corticosteroid hormone.

*A Comparison of the Effects on Man of Glucose Administration and Pursuit-Meter Stress.* We have previously presented evidence that the stresses of heat and pursuit-meter operation lead to ketosteroiduria and lymphocytopenia in normal men.<sup>3-6</sup> Using other possible indices of adrenocortical function, we have compared the effects of the Exton-Rose glucose tolerance test and the operation of the Hoagland-Werthessen pursuit-meter. FIGURE 4 presents the data thus far obtained with eight normal men who were given a sugar tolerance test and who, one week later, operated the pursuit-meter. Both tests were taken under basal conditions. The blood and urine data of FIGURE 4 are presented as percentages of the pre-stress values.

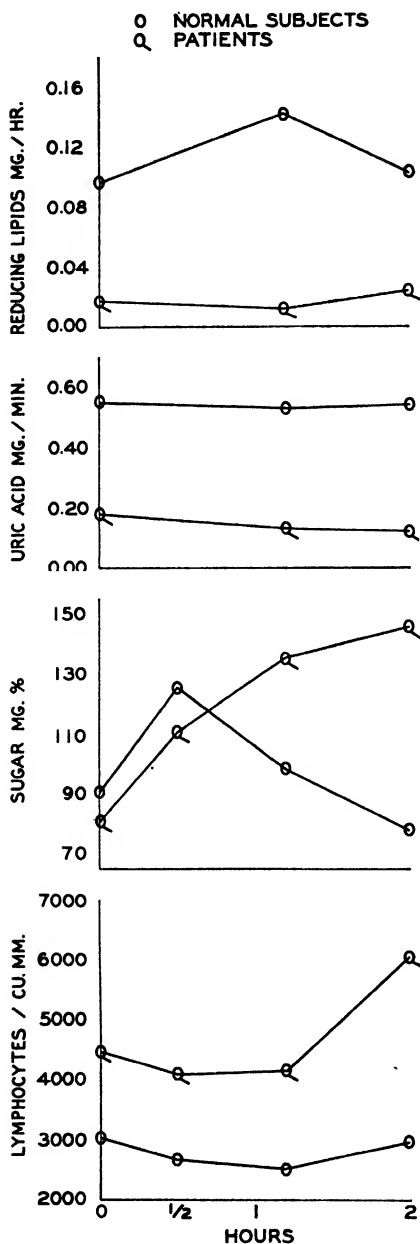


FIGURE 3. Mean variations in blood lymphocyte counts in blood sugar, urinary uric acid, and neutral reducing lipids in 5 normal subjects and three Addisonian patients in the course of Exton-Rose sugar tolerance tests.

The significant features of these data are: (a) both sugar administration and pursuit-meter operation are accompanied by significant increases in

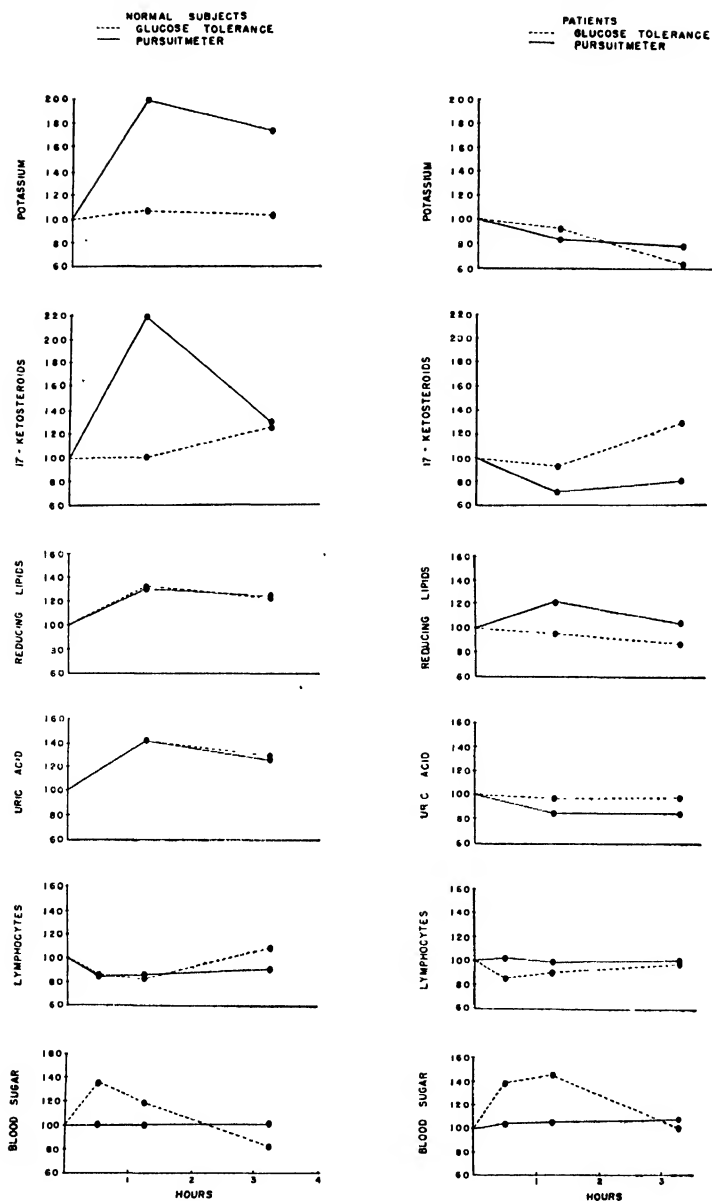


FIGURE 4

FIGURE 5

FIGURE 4 (Left-hand column). Mean variations in blood sugar and lymphocytes and in urinary potassium, 17-ketosteroids, neutral reducing lipids, and uric acid in eight non-psychotic men during and after glucose administration (---) and pursuit-meter operation (—). Each test lasted for one hour.

FIGURE 5 (Right-hand column). Data as in FIGURE 4, but for fifteen psychotic men.

neutral reducing lipid outputs; (b) the urinary uric acid increases significantly after the sugar administration and after pursuit-meter stress; (c)

the 17-ketosteroid output shows the characteristic rise, with a subsequent drop, previously described for pursuit-meter operation,<sup>3</sup> whereas, after sugar administration, the 17-ketosteroid output rises to some extent in the post-stress period; (d) the initial extent of blood lymphocyte decline is about the same after both the sugar and pursuit-meter tests, but there is a continued low level after pursuit-meter operation not observed in the sugar tolerance data; (e) the mean blood sugar change accompanying pursuit-meter operation is insignificant and is not at all correlated with the lymphocyte changes; and (f) there is a marked sustained increase of potassium excretion following pursuit-meter operation, whereas sugar administration leads to only slight change.

These data are consistent with the notion that sugar administration evokes the secretion of sugar-active corticosteroids, whereas pursuit-meter operation evokes a secretion of electrolyte-controlling hormone and 17-ketosteroid precursors as well. In fact, there is even the suggestion that the 17-ketosteroid precursor production is inhibited by sugar administration.

*Glucose and Stress in Psychotic Men.* We have data on fifteen schizophrenic men subjected in alternate weeks to the glucose tolerance and pursuit-meter tests. The mean data for these subjects are presented in FIGURE 5. It is clear that these men, on the average, do not exhibit the sort of responses shown by the non-psychotic men: (a) the neutral urinary reducing lipids show no increase in response to sugar and a small rise after pursuit-meter stress; (b) the uric acid output fails to show any increase in either situation; (c) the 17-ketosteroid output declines in both tests; (d) there is no notable lymphocytopenia in either test, although a large mean change in blood sugar is shown in the tolerance test and a small average increase occurs in the pursuit-meter data; and (e) after sugar administration and pursuit-meter stress, there is a continued decline in potassium excretion.

These data are consistent with the notion that the responsivity to either stimulative test of the adrenal cortex in the psychotic men is diminished or absent. Our original intention was to discover if there were any indications of differential reactivity in the psychotic subjects. The present data are inadequate for a detailed survey. However, Freeman and Elmadjian<sup>15</sup> have found that psychotic men given an Exton-Rose sugar tolerance test may be divided into two (nearly equal) groups: (a) those who show an inverse relationship between blood sugar and blood lymphocyte concentration (i.e., the relationship observed in normal men); and (b) those who show no inverse relationship, usually with no change in lymphocyte concentration. Actually in the data here presented, a similar division of individuals may be made, but there is no consistent correlated change in the other measures of adrenocortical function.

It may be concluded that the psychotic men who fail to show lymphocytopenia after sugar or pursuit-meter stress have an unreactive lymphatic system. That this is not so is shown by data on such men receiving adrenal cortex hormone. In FIGURE 6, are presented data on such subjects and a similar group of normal controls. It is clear that the psychotic men receiving adrenal cortex extract-in-oil react with a lymphocytopenia as extensive as that of the normal men receiving the same dosage.

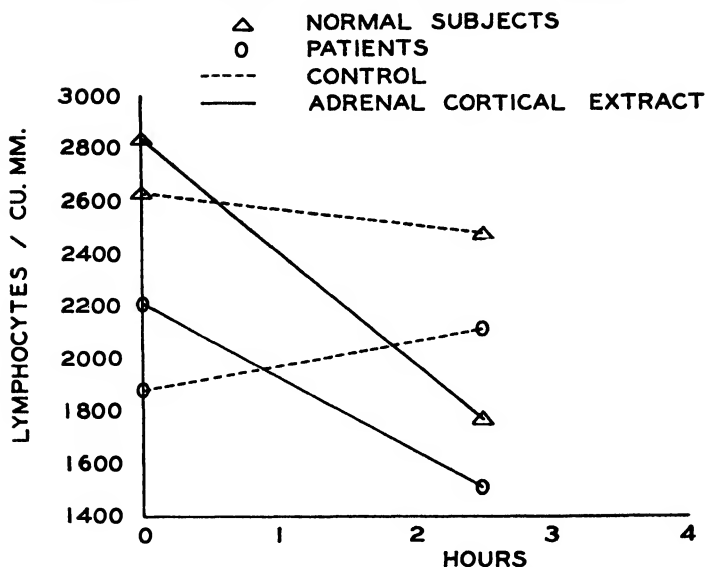


FIGURE 6. Five normal subjects and seven psychotic men received no medication on a control day (---) and 10 cc. of Lipo-adrenal extract (Upjohn) on an experimental day (—). Note that the lymphocytopenia on the experimental day is approximately the same in both groups of subjects.

*Adrenocortical Function in Psychoneurotic Subjects.* We have previously demonstrated that there is a diurnal rhythm of blood lymphocyte concentration in normal men and women.<sup>16</sup> Since this is a fairly regular increase from waking onward, and the obverse of the 17-ketosteroid rhythm,<sup>17</sup> we concluded that the adjustment to waking life involved, initially, a large secretory drain on the adrenal cortex followed by declining secretory activity through the day. Psychotic men generally appeared to show a flatter diurnal rhythm, i.e., less change of activity with waking and through the day.

We have similar data on the changes in blood lymphocytes occurring in psychoneurotic men and women. In FIGURE 7, are presented the mean curves relating lymphocyte number to time of day in these subjects compared with similar curves for normal and psychotic subjects. These graphs demonstrate: (a) that the absolute lymphocyte counts tend to be higher in psychotic subjects than in normal subjects, whereas in psychoneurotic subjects they tend to be lower, especially in the women with hysteria; (b) that the rate of change in lymphocyte number in psychoneurotic subjects is markedly reduced compared with normals. The latter finding is further amplified in TABLE 1, where the mean percentage change in lymphocyte number per hour is given for various groups of subjects. By calculating percentage changes, we obviate differences in absolute levels which are characteristic for each individual. It may be seen that the normal hourly rate of diurnal increase in blood lymphocytes is decreased by about 20 per cent in the male anxiety cases, by 48 per cent in the female anxiety cases and by 73 per cent in the hysterics. Statistically significant decline is had only for the hysterics, but the implication is that the stresses of daily life evoke frequent adrenal responses in the psychoneurotic subjects. They

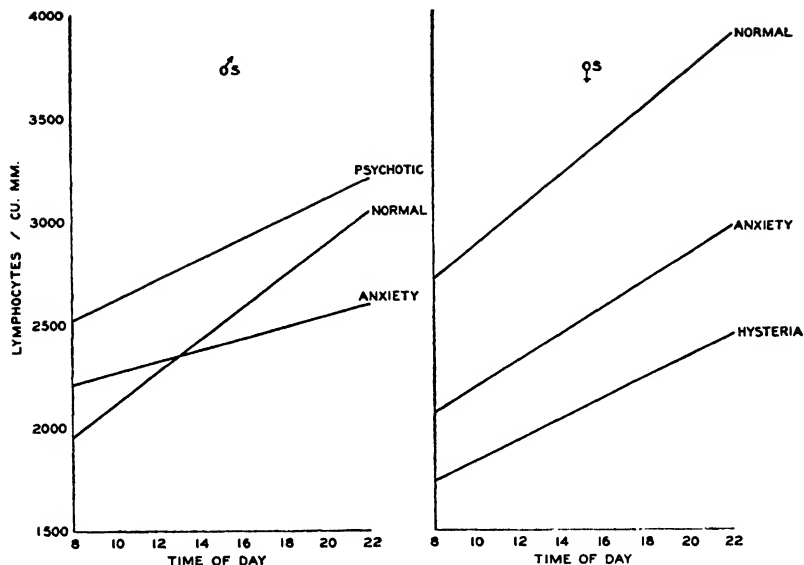


FIGURE 7. The regression of lymphocyte number on time of day for men and women of various mental types.

TABLE 1  
MEAN PERCENTAGE CHANGE IN CIRCULATING LYMPHOCYTES FROM WAKING TO SLEEP IN  
VARIOUS GROUPS OF SUBJECTS

Type of subjects	Sex	Number subjects	Number of daily observations	Mean % change in lymphocyte number per hr.	Coefficient of variation %
Normal	♂	13	51	$2.26 \pm 0.485$	148.2
Anxiety neurosis	♂	9	48	$1.80 \pm 0.518$	199.4
Normal	♀	13	40	$2.57 \pm 0.615$	175.9
Anxiety neurosis	♀	6	24	$1.35 \pm 0.923$	334.8
Hysteria	♀	8	39	$0.70 \pm 0.719$	641.4

tend not to let the adrenals rest. Furthermore, the high values of the coefficients of variation in the psychoneurotic groups indicate large fluctuation in the circulating lymphocytes.

We have a limited amount of data on the effect of certain stressful procedures on the circulating lymphocytes of psychoneurotic subjects. These data are presented in TABLE 2. The male patients were anxiety neurosis cases, the female patients chiefly hysteria cases. Again, the lymphocyte changes in the male patients are not significantly different from those in the control subjects, but, at one and three hours, the female patients have significant sharp drops in blood lymphocyte concentration. They appear to over-react to the stress.

Five of the psychoneurotic women were interviewed for one hour by a psychiatrist and indifferent matter discussed. On a succeeding day, an interview involving considerable probing and psychological stress was had.

TABLE 2

MEAN PERCENTAGE CHANGE IN CIRCULATING LYMPHOCYTES OF VARIOUS SUBJECTS BREATHING AIR CONTAINING 9 PER CENT O<sub>2</sub> FOR 30 MINUTES

(Room air was breathed through a mask for the first 30 minutes, 9 per cent O<sub>2</sub> introduced for the next 30 minutes, and the mask removed at the end of the hour.)

Type of subject	Sex	Number	Mean % decrease in lymphocytes @		
			½ hour	1 hour	3 hours
Normal	♂	5	15.5	11.4	13.0
Psychoneurotic.	♂	6	16.1	19.9	8.1
Psychoneurotic	♀	5	0.4	28.8	35.1

The blood lymphocyte changes occurring are presented in FIGURE 8. It is quite clear that neither immediately after nor some time after the non-

O - NON-STRESS INTERVIEW  
Q - STRESS INTERVIEW

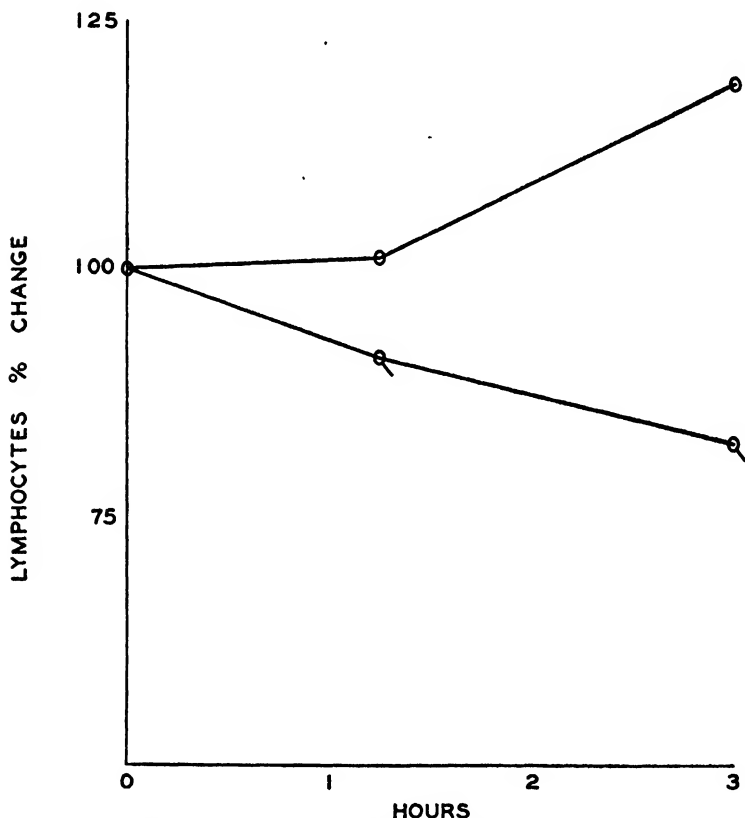


FIGURE 8. The effects on blood lymphocyte number of an interview in which indifferent matter was discussed (non-stress) and of an interview designed to create mental tension (stress). Data on five psychoneurotic women.



stress interview did any lymphocyte decrease occur, whereas progressive lymphocyte fall followed the stressful interview.

*Discussion.* The foregoing data demonstrate that means for measuring adrenal cortex responses in man are readily available. Furthermore, it is possible to differentiate responses evoked by different stimuli. Thus, in normal men, glucose administration evokes an increase in neutral reducing lipids in the urine but a decrease of 17-ketosteroids, whereas pursuit-meter operation evokes an increase of both. Increased potassium excretion and lymphocytopenia follow the pursuit-meter stress, but only the lymphocytopenia follows the sugar tolerance test.

By applying these tests to psychotic men, we obtain unequivocal evidence of a marked damping or absence of adrenal cortex response. The point of origin of this block in the alarm reaction is not evident from these data. It seems probable that the hormone target organs are not refractory, since patients respond like normal subjects to adrenal cortex extract administration. That the hormone-secreting mechanism of the adrenal cortex is at fault does not seem likely, since (a) the psychotic patients give no evidence of Addisonian levels of hormone secretion or excretion and (b) about half of them respond with lymphocytopenia to sugar administration. The responsiveness of the adrenal cortex in these psychotic men can be tested by administering adrenocorticotrophin, and, in a few preliminary experiments, completely normal reactivity has been found in some men but no responsiveness in others. It is our intention to determine the source of the block by proceeding to examine pituitary reactivity and thence the central nervous controls of the pituitary.

The data on psychoneurotic subjects thus far assembled indicate no particular block in the adrenal cortex activation mechanism. Rather, there are indications of overactivity of the adrenal cortex. This needs to be substantiated by further analytical data. The indications of rather large fluctuations in adrenal cortex activity in these cases implies a very labile reactivity. It may be that the crux of the psychoneurotic stress-responsivity lies in the source of this lability. Is it in the adrenal cortex itself?—in the pituitary?—in the hypothalamic control of the pituitary?

In presenting these beginnings of a systematic analysis of mental disease in terms of hormonal dysfunction, cautious interpretation is mandatory. Gross endocrinopathy is clearly absent, but it is perhaps noteworthy that those hormonal mechanisms involving homeostatic adjustment are the ones that seem to be malfunctional.

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# CLINICAL STUDIES IN ADDISON'S DISEASE\*

By GEORGE W. THORN, PETER H. FORSHAM, F. T. GARNET PRUNTY,†  
GRACE E. BERGNER,‡ AND A. GORMAN HILLS

*Department of Medicine, Harvard Medical School, the Medical Clinic, Peter Bent  
Brigham Hospital, Boston, Massachusetts*

## *General Considerations*

In following a series of one hundred and fifty-eight cases of Addison's disease,<sup>1</sup> it is apparent that two outstanding changes have occurred during the past decade:

1. There has been a marked decrease in the proportion of patients with tuberculosis as the etiology of Addison's disease; *i.e.*, tuberculosis is responsible now for adrenal insufficiency in only half the authors' patients.

2. There has been a considerable prolongation of life. Whereas, in the past, 80 per cent of patients with Addison's disease died within two years,<sup>2</sup> 50 per cent of the patients in the authors' series have survived a seven-year period of treatment (FIGURE 1). This remarkable improvement in life expectancy appears to be due in large measure to (a) the extensive use of synthetic desoxycorticosterone acetate,§ particularly as pellets implanted subcutaneously, combined with the use of whole adrenal cortical extract in emergencies when food intake is not adequate, and (b) the decrease in the incidence of tuberculous etiology, with its extremely unfavorable effect on the general management and prognosis of Addison's disease and the always present danger of a recurrence of activity.

With an increase in the period of survival, it is becoming steadily more important to improve the physical and mental status of these patients to a point where they can become self-supporting, or at least not remain a complete burden to society. The availability of 11-oxy-steroids in addition to desoxycorticosterone would appear to be a prerequisite in this endeavor.

## *Diagnostic Tests*

The diagnosis of classical cases of Addison's disease presents little difficulty and may be suspected on the basis of typical signs and symptoms such as those observed in one hundred patients in the authors' series (TABLE 1). In general, Addison's disease should be considered in: (1) patients with unexplained weight loss and asthenia; (2) patients with unexplained gastro-intestinal symptoms, especially in the presence of tuberculosis; and (3) a rare group characterized by manifestations of a neurological disease, including syncopal attacks and seizures occasionally accompanied by focal electroencephalographic changes.

Reduced heart size and hypotension are present in most instances. A normal heart size in an untreated patient with adrenal insufficiency suggests concurrent primary heart disease or hyperthyroidism.

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‡ Commonwealth Fund Fellow.

§ We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., for the Percorten pellets and solution used in these studies.

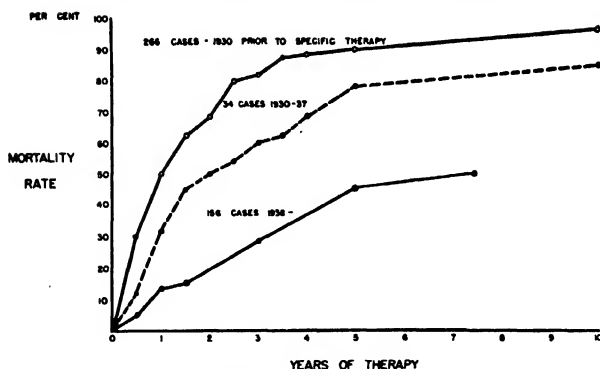


FIGURE 1. Mortality rate in Addison's disease. (From THORN, G. W., P. H. FORSHAM, & K. EMERSON, JR. 1949. American Lecture Series. Charles C. Thomas. Springfield.)

TABLE 1  
INCIDENCE OF SIGNS AND SYMPTOMS IN ADDISON'S DISEASE  
(100 patients)

Weakness and fatigability	100%
Weight loss	100%
Increased pigmentation	94%
Anorexia, nausea, and vomiting	85%
Diarrhea	21%
Salt-craving	19%
Muscle pain	16%

Hypometabolism (minus 10 to minus 15 per cent) is typical of the disease. A basal metabolic rate of zero or above usually indicates complicating hyperthyroidism. The classical signs and symptoms of the latter may appear minimal in spite of a definite enlargement of the thyroid and continued weight loss. Conversely, a basal metabolic rate below minus 25 per cent suggests coexisting hypothyroidism. With gonadal insufficiency, it suggests hypopituitarism as the cause of both adrenal insufficiency and the other deficiencies. This condition is characterized by the paucity of pigmentary abnormalities and a relative preponderance of hypoglycemia as opposed to hypochloremia.

In more advanced cases of Addison's disease, one may expect hemoconcentration and a reduction in serum sodium and chloride level, with a rise in serum potassium. The ratio (normal = 30) of sodium to potassium will be decreased.

Measurement of the twenty-four hour urinary excretion of 17-ketosteroids is a valuable diagnostic aid (TABLE 2). Unfortunately, many non-specific

TABLE 2  
URINARY 17-KETOSTEROID EXCRETION (MG. PER DAY) IN 70 PATIENTS WITH ADDISON'S DISEASE

	Range	Mean	Mean Deviation
Males (37)	1.0-8.0	4.2	$\pm 2.7$
Females (33)	0.1-8.6	3.0	$\pm 2.0$

Normal means for males, 15.0; for females, 10.0.

conditions may result in a low 17-ketosteroid excretion. This is particularly true of diseases associated with a marked inanition. On the other hand, a normal 17-ketosteroid excretion value is reliable in *ruling out* Addison's disease.

The establishment of milder or earlier degrees of adrenal cortical deficiency may be extremely difficult and will require, in most instances, the use of tests designed to place the adrenal cortex under stress. Such tolerance tests may be applied to most of the known functions of the adrenal cortex. The Robinson-Kepler-Power water test<sup>3</sup> for water diuresis is a perfectly safe screening test, but tends to give a positive response in patients with kidney disease, liver disease, and certain abnormalities of the gastro-intestinal tract, as well as in Addison's disease.

The Cutler-Power-Wilder test for sodium retention<sup>4</sup> is valuable, and a positive response is highly significant, but with the marked dehydration and potassium retention that may ensue there is considerable risk of an adrenal crisis. The test requires careful dietary management throughout and should be employed in a hospital under strict observation.

Tolerance tests based on the derangement in carbohydrate and protein metabolism are designed to reveal an inability to preserve or replenish carbohydrate levels once depressed by various means. The induction of hypoglycemia at the end of a glucose tolerance test (FIGURE 2), after the twentieth hour of a twenty-four hour fast (FIGURE 3), or following the administration of a small dose of insulin, not only aids in the diagnosis, but assists in assessing the need for a high carbohydrate diet with frequent feedings, supplementary whole adrenal extract therapy, or both.

Unfortunately, the application of any of these tests represents a real hazard to the patient. Following the intravenous glucose tolerance test, as well as the administration of excessive amounts of intravenous glucose, we have observed on many occasions a characteristic syndrome conveniently termed "glucose fever." After an essentially uneventful glucose tolerance test, with or without hypoglycemic manifestations, which may occur at blood sugar levels as high as 60 mg. per cent on occasion, all appears well. Some twelve hours later, the patient suddenly experiences a chill which is followed by marked hyperpyrexia (FIGURE 4). Usually, several waves of fever appear, and a normal temperature is reached again after two or three peaks. Malaise, aches and pains, nausea and vomiting, and varying degrees of disorientation accompany these episodes. Although a careful search is made for sources of infection, bacteriological studies are negative, and pyrogens cannot be demonstrated as a rule in the solutions used intravenously. Administration of whole adrenal extract supplemented with plasma or albumin has proved life-saving in treating patients with this complication. The similarity of this reaction to the manifestations commonly associated with water intoxication and the rather dramatic improvement following the administration of human albumin have led the authors to consider this reaction a form of abnormal cellular edema involving the central nervous system.

Recently, the use of pituitary adrenocorticotrophic hormone (ACTH)\*

\* We are indebted to Dr. John R. Mote of the Armour Laboratories for the purified pituitary adrenocorticotrophic hormone used in these studies.

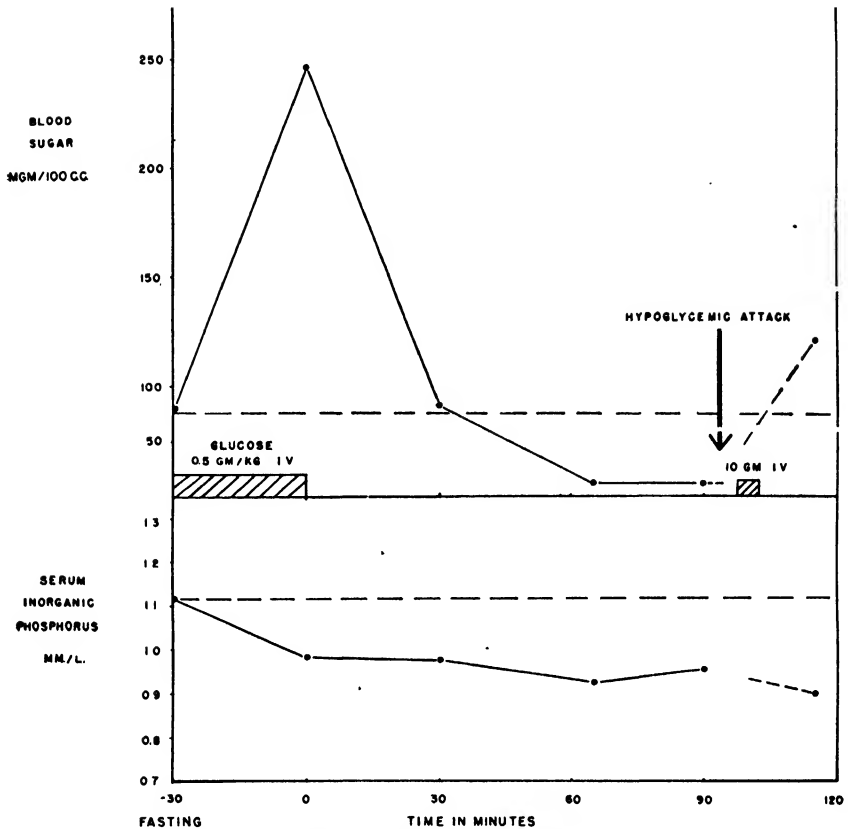


FIGURE 2. Intravenous glucose tolerance curve, Addison's disease. Patient M. N. ♀, age 45.

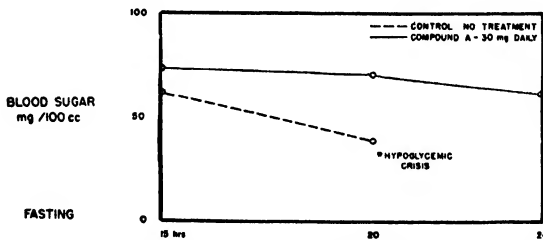


FIGURE 3. Blood sugar during prolonged fast, Addison's disease. Patient V. A. ♀, age 37. (From FORSHAM, P. H., G. W. THORN, G. E. BERGNER, & K. EMERSON, JR. 1946. *Am. J. Med.* 1: 105.)

has been found to be of considerable aid in testing for the presence of a normal adrenocortical response. Studies on patients with an intact adrenal cortex have shown that many metabolic changes follow the administration of ACTH. There is an increased nitrogen output, increased uric acid excretion, a decreased sodium output, an increased fasting blood sugar level, an increased 17-ketosteroid and 11-oxy-steroid excretion, and a decrease in circulating lymphocytes and eosinophils, with a variable leucocytosis. The

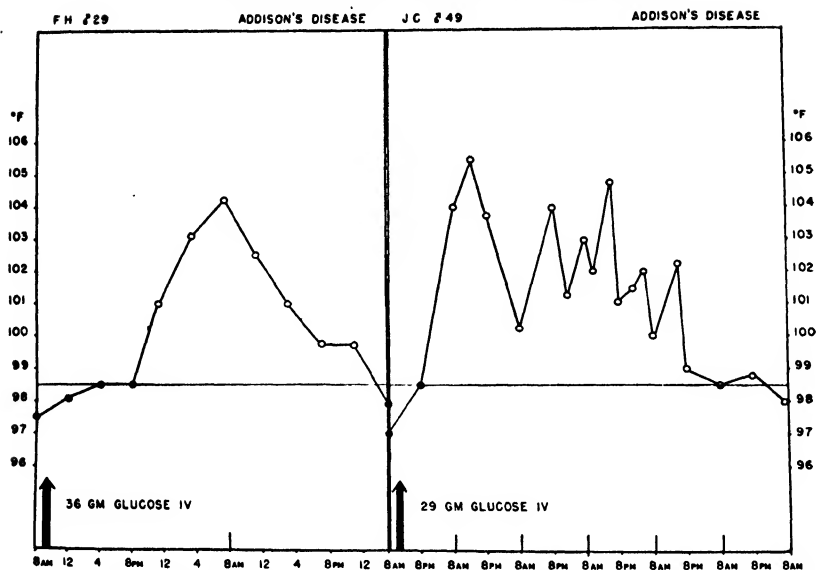


FIGURE 4. Febrile response following IV-glucose administration.

most characteristic and easily measured changes were the increase in the uric acid, conveniently expressed in terms of the urinary uric acid-creatinine ratio, and the decrease in circulating lymphocytes and eosinophils. Direct enumeration of eosinophils was employed, using a Levy counting chamber and an eosin-acetone-water diluting fluid.<sup>5</sup> The average response of patients with Addison's disease to intramuscular ACTH as judged by any of the above changes fell far below that of normal subjects and miscellaneous hospital patients. That this was due to an inability of the adrenal cortex to increase further the output of cortical hormones and not to an abnormality in the various end-organs, was proved by experiments such as that shown in FIGURE 5. In the case of the uric acid-creatinine ratio (FIGURE 6) and the decrease in eosinophils (FIGURE 7), no patient with Addison's disease manifested a response to ACTH as great as that of the least responsive subject with normal adrenal cortical reserve.

On the basis of these findings, a simple clinical test has been devised. The patient is fasted overnight, and urine is collected from 6:00 a.m. to 8:00 a.m. At 8:00 a.m., 25 mg. of ACTH in saline is administered intramuscularly. A second urine collection is made from 9:00 a.m. to 12:00 noon, and then the patient is given lunch. Blood elements are determined at 8:00 a.m. and at 12:00 noon, the uric acid-creatinine ratio is estimated in both urine samples, and the per cent increase from the early to the late specimen is calculated. Renal insufficiency may mask the urinary response. The hematological changes, however, will still show the characteristic difference between the normal subject and patients with primary adrenal cortical insufficiency.

In testing for mild forms of adrenal cortical insufficiency, a therapeutic

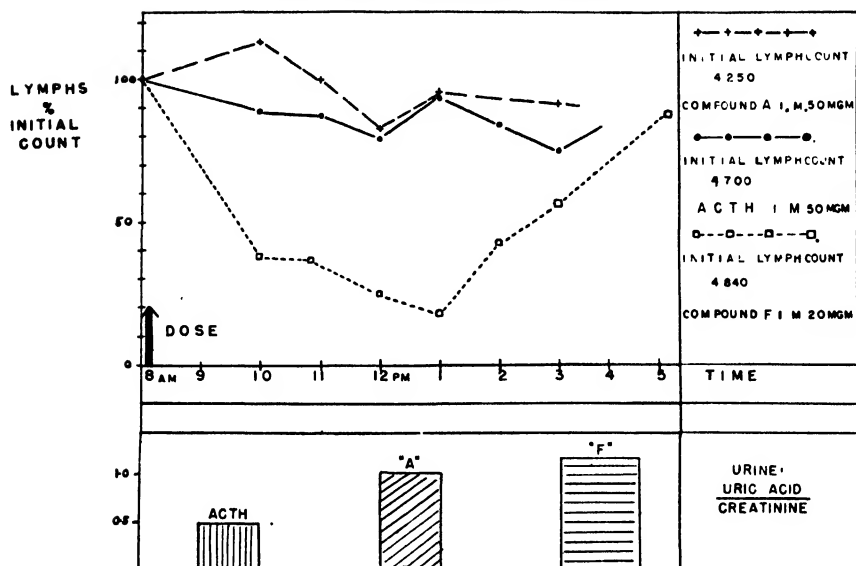


FIGURE 5. Effect of single doses of ACTH and compounds A and F in Addison's disease. Patient C. S. 9

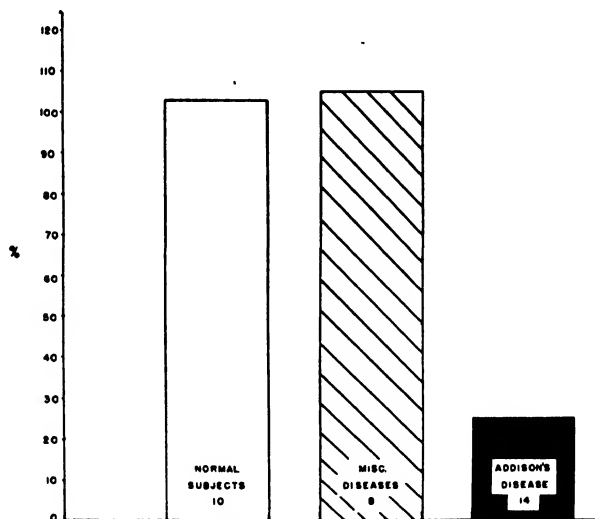


FIGURE 6. Increase in  $\frac{\text{uric acid}}{\text{creatinine}}$  ratio following ACTH administration.

trial with desoxycorticosterone acetate forms a safe and satisfactory method of diagnosis. The test as performed on an ambulatory patient is as follows: Weights taken prior to breakfast are recorded, and daily basal blood pressures are obtained whenever possible. The patient is told not to make any major change in the usual salt or fluid intake. Hematocrit is determined initially and again at the termination of ten days of Percorten therapy, 0.5



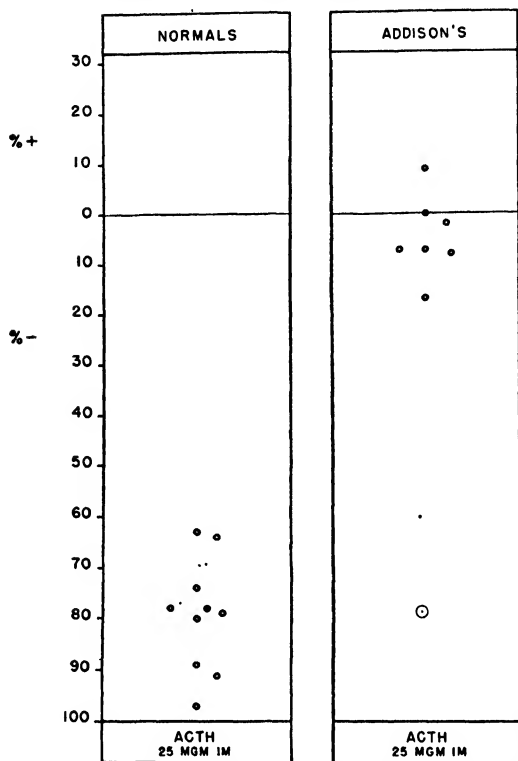


FIGURE 7. Per cent change of eosinophil count four hours after ACTH. The point • represents the fall in eosinophils observed in one of the patients with Addison's disease given 20 mg. of Compound F intramuscularly.

cc. (2.5 mg. desoxycorticosterone acetate intramuscularly) daily. Changes in weight, blood pressure, hematocrit, and clinical response are followed. This trial is repeated using 0.5 cc. of sesame oil (placebo) for ten days, and the Percorten is then resumed.\* In patients with Addison's disease, there will be clinical improvement, weight gain, hemodilution, and possibly a slight increase in blood pressure under Percorten therapy, with regression upon institution of placebo injections. Neurasthenics will show only insignificant physiological responses and will continue to feel better during the placebo period.

### *Treatment*

The treatment of Addison's disease is an example of substitution therapy which has become more specific over the years. The milestones in the use of hormonal preparations, isolated or synthesized, are presented in TABLE 3.

It is now generally accepted that the following abnormalities exist in adrenal insufficiency:

Decreased sodium and chloride retention.

\* Ciba Pharmaceutical Products, Inc., provides a diagnostic package which contains both Percorten solution and placebo sesame oil.

TABLE 3  
HISTORICAL DEVELOPMENT OF HORMONE THERAPY

- 1895. Use of glycerine extract of fresh adrenals by Osler.
- 1929. Preparation of active adrenal extracts by Hartmann, Rogoff, and Swingle.
- 1938. Partial synthesis of desoxycorticosterone by Reichstein.
- 1943. Purified porcine extract in oil with a high 11-oxy-steroid content (Upjohn).
- 1946. Partial synthesis of dehydrocorticosterone by Kendall and by Reichstein.

Diminished carbohydrate preservation and synthesis from protein.

Inadequate utilization of fat.

Moderate impairment of androgenic functions.

Decreased lympholysis and possibly a decreased release of gamma globulin and antibodies.

Characteristic pigmentation.

The importance of supplementary sodium chloride therapy in the treatment of Addison's disease, as suggested by Loeb<sup>6</sup> and Harrop,<sup>7</sup> has been substantiated by continued clinical experience and is an effective means of reducing the cost of specific treatment in those patients requiring hormone.

Experience in the use of desoxycorticosterone acetate, with its powerful sodium chloride retaining action, has clearly shown that for patients treated with this synthetic hormone a low potassium diet is neither desirable nor helpful and is, indeed, contra-indicated in most instances. Supplementary sodium chloride is to be used with great caution in conjunction with desoxycorticosterone. By regulating the dosage carefully, it has been possible to avoid many of the undesirable side effects found more frequently in the earlier period of treatment<sup>1</sup> and to improve greatly both the clinical condition and life expectancy of patients with Addison's disease. Over the past nine years, pellets of desoxycorticosterone acetate, implanted subcutaneously, have been used with success in over two hundred patients. This method obviates the necessity for daily injections of hormone and eliminates the possibility of allergic reactions to the oily medium in which the synthetic hormone is dissolved. The average number of pellets implanted (weight 125 mg.) in one hundred of our patients between January, 1944, and September, 1946 has been four for males and three for females, approximately once a year. Although hypertension is frequently reported as a complication to desoxycorticosterone acetate therapy, the mean blood pressure of fifty patients recently admitted to the Peter Bent Brigham Hospital for implantation of pellets has been 114/17 mm. Hg at the time of entry, and 125/81 mm. Hg one to six months after implantation. Heart size, below normal in all uncomplicated cases, has been brought back to, but not above, normal after treatment in fifty cases recently analyzed. Thus, hypertension, edema, and heart failure have been practically eliminated as complications of overdosage with desoxycorticosterone, but the occurrence of arthralgia and tendon contraction is still a debilitating complication which arises in an occasional case. The treatment includes the administration of potassium citrate, a reduction in the sodium chloride intake, and administration of small amounts of prostigmine to release painful contractures.

Over one-half of the authors' patients do well on desoxycorticosterone

acetate supplemented by a high caloric diet. Since desoxycorticosterone fails, however, to correct the disturbances in carbohydrate, protein, and fat metabolism which exist in this disease, supplementary whole adrenal extract therapy has been used whenever patients show signs of weakness and hypoglycemia and experience frequent bouts of non-specific infections when on the standard type of therapy. The very high cost makes this type of therapy available to the vast majority of the authors' patients only for periods of inadequate food intake, medical emergencies, and surgical intervention.

Preparations showing carbohydrate regulating activity are shown in TABLE 4. Bovine extract in aqueous solution has a short period of action

TABLE 4

## ADRENAL CORTX PREPARATIONS POSSESSING CARBOHYDRATE REGULATING EFFECT

1. Whole extract (bovine) in aqueous solution.
2. Whole extract (porcine) in oil.
3. Synthetic 11-dehydrocorticosterone (Compound A, Kendall).
4. Naturally occurring 11-oxy steroids (Compounds A, B, E, and F).

(four hours) with an early onset and lends itself to use in acute emergencies when at least 50 cc. per day must be given intramuscularly.

The preparation of a purified adrenal extract standardized in terms of crystalline steroid hormone for its carbohydrate regulating activity (Lipo-Adrenal Cortex, Upjohn),\* has been a great advance in the treatment of patients with Addison's disease. The longer continued action of this hormone preparation in oil (eight hours) reduces the number of injections per day.

Unfortunately, adequate supplementary extract treatment, either 10 to 20 cc. of adrenal cortex aqueous preparation daily or 2 to 3 cc. of Lipo-Adrenal Cortex daily, raises the cost of treatment to approximately \$2500 yearly; whereas desoxycorticosterone acetate treatment alone (pellets implanted subcutaneously or daily intramuscular injections of hormone in oil) costs approximately \$100 yearly.

In crises and in acute emergencies, adrenal cortical extract has proved life-saving in many of the authors' cases. It is important to differentiate between a crisis occurring in a patient under treatment with desoxycorticosterone acetate, and therefore usually well-hydrated, from a crisis occurring in a patient who has never received treatment. In the latter case, the use of large quantities of sodium chloride parenterally is of great benefit; whereas excessive administration of salt and water to a patient on desoxycorticosterone acetate treatment may lead to fatal overhydration. The authors have used large quantities of whole adrenal extract, however, in the treatment of crisis induced by infection or trauma in a patient under desoxycorticosterone acetate therapy. Salt retaining equivalents are presented in TABLE 5. It has also been possible with adequate supplementary extract therapy to carry patients with Addison's disease through major surgical procedures.

Interest and hope are aroused by the recent synthesis of an 11-oxy adrenal

\* We are indebted to Dr. E. Gifford Upjohn of The Upjohn Company for the Lipo-Adrenal cortex used in these studies.

TABLE 5  
APPROXIMATE EQUIVALENTS  
Sodium-Chloride Retaining Effect

Preparation	Dose	Administration
Desoxycorticosterone acetate in oil	1 mg.	Once daily I.M.
Pellets of desoxycorticosterone acetate (125 mg.)	2	q 9-12 mos.
Adrenal extract (bovine)*	5 cc.	q 4 hrs. I.M.
Lipo-Adrenal cortex (porcine)*	1 cc.	q 8 hrs. I.M.

\* Equivalence in carbohydrate regulating capacity is the same for these preparations.

steroid, Compound A,\* according to the method of Kendall. Although it is doubtful that this preparation or its more potent relatives, Compounds E and F, will be available commercially for some period of time, it may be appropriate to record the authors' limited experience with this Compound,<sup>8</sup> which differs in some respects from the findings reported by others.<sup>9</sup>

Fourteen patients with classical Addison's disease have been treated with this preparation. With a daily dose of approximately 30 mg., a small decrease in sodium and chloride excretion was observed in conjunction with a moderate increase in the renal excretion of total nitrogen, inorganic phosphorus, and potassium. Compound A appeared to be approximately one-twentieth as active as desoxycorticosterone acetate in its sodium retaining capacity and less than one-half as active as Compound F in carbohydrate regulating potency. The most outstanding effect which was observed under Compound A therapy was the ability of patients to withstand a twenty-four hour fast which under control conditions resulted in a severe hypoglycemic reaction (FIGURE 3).

In metabolic experiments with Compound A, it was evident that clinical improvement could not be ascribed entirely to glucose derived from protein (increased gluconeogenesis), since the increased quantity of nitrogen excreted was so small. The increase in uric-acid excretion, which was observed in thirteen of the fourteen patients who received Compound A, was a more constant finding than the slight increase in nitrogen excretion observed. There was no detectable fall in circulating lymphocytes.

The addition of Compound A therapy to a basic regimen of desoxycorticosterone acetate resulted in clinical improvement in the four patients with severe Addison's disease to whom it was possible to administer 20 to 30 mg. daily for periods of two to three months. Regression followed rapidly the withdrawal of Compound A.

It is of interest that none of the therapeutic measures employed to date have truly altered the state of pigmentation in the authors' patients beyond a generalized lightening associated with rehydration and an unexplained, slight, but definite decrease in generalized tan upon the long-continued administration of Compound A.

\* We are indebted to the late Dr. D. F. Robertson of Merck & Co., Inc. for the Compound A used in these studies.

*Conclusions*

The diagnosis of adrenal cortical insufficiency has been greatly improved over the past decade and tests are available today whereby practically every known function of the adrenal cortex may be evaluated separately. It would appear that clinical differences within the same disease process will be confirmed by finding unequal impairment of various functions in what grossly appears to be the same disease. With recent advances in the specific replacement therapy, survival of patients with Addison's disease has been improved greatly. It is apparent, however, that further improvement in mortality rate and in clinical conditions will depend upon the availability of 11-oxy-steroids for use in conjunction with a basic maintenance dose of desoxycorticosterone acetate. In the absence of active tuberculosis, such amplified replacement therapy should make it possible for patients with Addison's disease to lead a useful and independent existence.

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# CUSHING'S DISEASE: A PRIMARY DISORDER OF THE ADRENAL CORTICES?

By EDWIN J. KEPLER\*

*Division of Medicine, Mayo Clinic, Rochester, Minnesota*

Cushing's disease was selected as a subject with the hope that it might appeal to the experimentalist as well as to the clinician who is interested in endocrinology. Some of the data previously have appeared in print. More will be reported elsewhere. At the present time, repetition of this sort seems to be unavoidable.

Literature pertaining to Cushing's syndrome has accumulated with a rapidity suggestive of a geometric progression, whereas my ability to read it proceeds arithmetically. Consequently, I shall review comparatively little of it. Instead, I shall restrict myself to a summary of impressions that I have gained from reading a portion of it. This portion, I hope, has been representative.

By necessity, I shall draw heavily from my own personal experience, which, because of my position, has been relatively extensive. It is necessary to use the word "relative" because Cushing's disease occurs infrequently, and consequently no one person ever has the opportunity to study a large series of cases. Its incidence is not known. In the larger cities, patients afflicted with it occasionally may be seen on the streets and in public places. In smaller communities, it is distinctly a rarity. For example, in the twenty-odd years that I have lived in Rochester, Minnesota, a town of about 35,000 people, to my knowledge not a single case has appeared locally. Had any such cases occurred, almost certainly the patient would have consulted the Mayo Clinic, since few patients in the city or its immediate environs seek medical advice elsewhere, once they become seriously ill.

To conclude these introductory remarks, I must emphasize the fact that few, if any, of the basic concepts that I have pertaining to the subject are original. They represent merely the end result of the intellectual contacts which I have had with immediate associates and scientific friends elsewhere.

It was in 1932 that Cushing<sup>1, 2</sup> published his original articles on basophilic tumors of the hypophysis. In them he called attention to an old but unusual "polyglandular" clinical picture, which, hereafter, I shall refer to as Cushing's syndrome.† Its salient features are: a distinctive habitus characterized by wasting of muscle and by obesity or abnormal distribution of fat, the combination of which makes the trunk, face, and neck appear obese and the extremities thin; hirsutism; hypertension; osteoporosis; diabetes, either latent or frank; ecchymosis; and amenorrhea or impotence. Usually, there is a pad of fat over the cervicothoracic portion of the spinal column. The eyelids and corners of the mouth droop, and, when the face is viewed squarely

\* Died October 19, 1947.

† The term "Cushing's syndrome" will be used to designate the clinical picture described by Cushing. The term "Cushing's disease" will be used to designate all cases presenting this clinical picture, except those associated with an adrenal cortical tumor. In other publications, I have employed the term to include only those cases in which the clinical picture was associated with a basophilic tumor of the anterior pituitary body.

from the front, the ears may be partially or totally concealed by the fullness of the face. Almost always there is an easily demonstrable muscular weakness, sometimes of a degree seen in moderately advanced muscular dystrophy or atrophy, so that in stepping up ordinary stairs the patient may have to use his hands to lift his feet. Acne of varying intensity is fairly common. Generally, the skin is thin and there are distinctive purplish striations. Polyuria occurs fairly frequently. Sometimes there is erythremia; nearly always there is lymphopenia. The concentration of the plasma potassium is reduced more frequently than has usually been reported, and occasionally a full-blown hypochloremic alkalosis is present, with or without elevation of plasma sodium. The amount of urinary 17-ketosteroids may be normal or increased moderately or greatly, depending on the pathologic processes responsible for the clinical picture. In the cases studied, assay of the urine for corticosteroids or chemical analysis for these substances, by the method developed by Talbot and his associates,<sup>3</sup> has disclosed increased values.<sup>4</sup> What combination of symptoms constitutes a *sine qua non* for making the diagnosis is a matter of opinion.

Cushing, in a series of cases, found at necropsy that the clinical picture just mentioned was associated with small basophilic tumors of the pituitary body. In other instances, he made an accurate anatomic diagnosis before death. Furthermore, he predicted correctly that if the pituitary body were re-examined in certain cases which had been reported in the literature, a basophilic adenoma could be demonstrated. He attributed the clinical picture to the secretory activity of these tumors. His reasoning is refreshing in its simplicity. Essentially, it was as follows: Three types of cells are found in the anterior pituitary body; namely, chromophobic cells which are nonsecretory, and eosinophilic and basophilic cells which secrete hormones. Any one of these three cell types may give rise to its own peculiar adenomatous formations. The chromophobe tumors, consisting of nonfunctioning cells, are destructive nonfunctioning lesions. Adenomas arising from the eosinophilic cells are functionally active and produce the clinical picture known as acromegaly. Adenomas arising from basophilic elements, theoretically, therefore, also should have functional activity. The secretory products of such basophilic adenomas in turn would affect the function of other members of the endocrine system, particularly the gonads, the adrenal cortices, and other loci as well. As a result, "a polyglandular syndrome hitherto supposed to be of cortico-adrenal origin might be brought about."

Immediately after Cushing's articles were published, further examples of the syndrome were reported and data on small series of cases began to appear in the literature.\* Simultaneously, two main schools of thought developed. Members of the first school emphasized the pathogenic importance of the pituitary body. Members of the second, exemplified, for example, by Kepler and his associates,<sup>14</sup> Bauer,<sup>15</sup> Kraus,<sup>16</sup> Anderson, Haymaker, and Joseph,<sup>17-19</sup> Albright,<sup>20</sup> and others listed by Luft,<sup>13</sup> for reasons to be shown subsequently in this paper, stressed the pathogenic potentialities of

\* Reviews will be found in articles by Freyberg and his associates,<sup>5</sup> Kessel,<sup>6</sup> Bland and Goldstein,<sup>7</sup> Tesseraux,<sup>8</sup> Haymaker and Anderson,<sup>9</sup> Eisenhardt and Thompson,<sup>10</sup> Kepler,<sup>11</sup> Thompson and Eisenhardt,<sup>12</sup> and Luft.<sup>13</sup>

the adrenal cortices. Evidence accumulated to support each of these divergent schools of thought. Some of it is based on observations made by clinicians and pathologists. The remainder consists of studies conducted on laboratory animals by workers engaged primarily in fundamental research.

In the material that follows, I shall review a few of the older observations, examine critically conclusions that have been drawn from them, and then present clinical data recently acquired by my associates and myself.

### *Evidence from Pathological Material*

Cushing's contention lost ground when it was shown that the small basophilic adenomas which he described could be found comparatively frequently when searched for routinely at necropsy.<sup>21-23</sup> Since the adenomas occurred so frequently and the clinical picture so rarely, it seemed entirely logical to conclude that the relationship of the adenoma to the clinical picture was fortuitous and not causative. This conclusion has always seemed faulty to me. Applying the same line of reasoning to adenomas of the ductless glands, one could conclude that adenomas of the islet cells of Langerhans never caused hyperinsulinism, that adenomas of the thyroid gland never caused hyperthyroidism, and that adenomas of the adrenal cortex never caused adrenogenitalism, since in all three instances the incidence of adenomas without clinical evidence of hyperfunction is greatly in excess of that of those with clinical evidence of hyperfunction. No clinical or surgical endocrinologist would be willing to accept this conclusion.

As Cushing anticipated, objections to his thesis were raised because the tumors he discovered were small, whereas the clinical disturbances that accompanied them seemed to be disproportionately great. Subsequently, however, it was demonstrated that not all of the tumors were small. In fact, some were large enough to erode the sella turcica and were associated with all the significant endocrinological abnormalities that he had mentioned.<sup>24-26</sup> In addition, as he pointed out in his original articles, size *per se* cannot be regarded as a criterion of adenomatous functional capacity. This lack of correlation between size and physiological disturbances is particularly evident in the case of parathyroid tumors and pancreatic islet cell adenomas.

Cushing's general thesis was both strengthened and weakened by observations made by Crooke.<sup>27, 28</sup> These observations have been confirmed repeatedly.<sup>12, 23, 29\*</sup> He examined the pituitary body in a series of cases of Cushing's syndrome and found, in each instance, the cytologic appearance of the basophilic cells altered in a peculiar and specific fashion. The cytoplasm was hyalinized, degranulated, and contained vacuoles. These changes were not found in the cells of the basophilic tumor, if one happened to be present, but were confined entirely to the cells in the non-tumorous portion of the gland. They were almost entirely limited to cases in which the clinical picture was that described by Cushing and occurred invariably in these instances, even when gross macroscopic lesions were found in other endocrine structures such as the adrenal cortices and the thymus. Crooke concluded

\* Kraus,<sup>16</sup> in 1937, and Severinghaus,<sup>30</sup> in 1938, commented on cells that were very similar to, if not identical with those described by Crooke.



that these microscopic alterations of the basophils were the pathological denominator common to all cases of Cushing's syndrome and that, therefore, they were the anatomic expression of the disturbed function of the basophilic cells which gave rise to the clinical picture. His concept obviously threw out the basophilic tumor as an etiologic factor, but still kept the anatomical and functional source of the disorder confined to the pituitary body and the basophilic cells. For reasons which have been published elsewhere,<sup>31</sup> this conclusion was not inevitable. The changes which he described can logically be regarded as a retrograde\* or degenerative lesion, secondary to disordered function and hyperactive in character, of the adrenal cortices. Kraus,<sup>16</sup> in 1937, came to a similar conclusion.

In many cases of Cushing's syndrome, hyperplastic or neoplastic lesions of the adrenal cortex have been discovered either at operation or at necropsy. The exact incidence of pathological changes in the adrenal cortex has not been determined, but some idea of their frequency can be obtained from two series of cases that have been analyzed recently. The first series, containing 98 cases, was collected by Thompson and Eisenhardt.<sup>12</sup> In it, there were 22 cases of adrenal cortical tumor. The second series was reviewed by Gorsuch.<sup>33</sup> From the literature, he selected seventy-two fatal cases in which the clinical picture conformed closely to that described by Cushing. He tabulated the gross pathological lesions as follows:

Neither pituitary nor adrenal pathological change in 7 cases.

Basophilic pituitary tumor without adrenal pathology in 11 cases.

Miscellaneous pituitary tumors (not basophilic) in 6 cases.

Adrenal cortical adenoma in 8 cases.

Adrenal cortical carcinoma in 7 cases.

Adrenal cortical hyperplasia in 9 cases.

Pituitary and cortical lesions in 24 cases.

Crooke's changes were not mentioned, but, presumably, they were present in most of the cases. Also, adrenal cortical hyperplasia, rightly or wrongly, was excluded if the smaller of the two adrenal glands did not weigh 10 gm. or more. It is evident that, in less than 22 per cent of Gorsuch's cases, pituitary lesions only were present, in one third, adrenal lesions only, and in another third, lesions were present in both organs. Fifteen cases of adrenal cortical carcinoma were excluded from this series of seventy-two fatal cases because some of the patients recovered and because the diagnosis was made by surgical exploration. In an appreciable number of instances of Cushing's syndrome, the adrenal cortices have been described as being normal in size and microscopic appearance, and in one case described by Freyberg and his associates<sup>6</sup> both adrenal cortices were thought to be "hypoplastic."† Adherents to the adrenal school of thought naturally have been impressed by this high incidence of adrenal cortical lesions and regard the so-called normal adrenal glands that are encountered as being examples of the shortcomings of histologic technique or interpretation.

\* Kraus<sup>32</sup> recently has reviewed elsewhere the various cytologic changes that occur in the anterior pituitary consequent to primary disease.

† These authors felt that the histologic appearance of the cells did not necessarily signify decreased secretory activity.

Advocates of the pituitary theory accept the high incidence of adrenal cortical lesions and regard them as being the result of intense stimulation of the adrenal cortices by the abnormal anterior pituitary body. In the case of malignant adrenal cortical tumors, they naturally have to assume not only stimulation by the basophilic cells of the pituitary body, but also the existence of those specific and predisposing factors which are responsible for the development of malignancy in general. Unilateral malignant adrenal tumors, particularly when associated with Cushing's syndrome, have often been found to be accompanied by functional and anatomical contralateral cortical atrophy. It is particularly difficult to adapt the pituitary theory to this unpleasant fact.\* The theory implies that a pituitary trophic agent could stimulate one adrenal cortex to a point where neoplastic changes could occur and simultaneously not only fail to stimulate the other adrenal cortex, but actually permit it to atrophy. Of course, one might argue that the contralateral gland was always atrophic and that compensatory hyperplasia was followed by neoplasia of the opposite side. Clinically, this explanation is not satisfactory, because the atrophic contralateral gland does not seem to be permanently incapacitated. Once the functioning adrenal neoplasm is removed, the contralateral atrophic gland rather promptly begins to function and presumably regenerates.

A few isolated pathological observations have been made which are difficult to account for by either theory. In a small number of cases of Cushing's syndrome, malignant lesions of the pancreas or of the thymus have been discovered at necropsy.<sup>34, 35</sup> In most of these cases, the adrenal cortices have been found to be hyperplastic.

I will consider the thymic cancer first. Because it happens to have been present, many authors have tacitly concluded that it must be a functioning tumor, which, in some mysterious fashion, produced the clinical picture. I say mysterious because, to my knowledge, there is no good evidence to indicate that the thymus secretes any hormonal products, and no evidence whatsoever that any hormones it might secrete could possibly produce the symptoms of Cushing's syndrome. For the time being, at least, the thymic tumors, therefore, can best be regarded as coincidental or secondary phenomena. Personally, I think they are probably secondary and not coincidental. Thymic tissue in cases of Cushing's syndrome is probably very active tissue, possibly because of the action of corticosteroids of the Compound E (11-dehydro-17-hydroxycorticosterone) type on lymphoid tissue. White and Dougherty<sup>36, 37</sup> have shown that the administration of Compound E caused involution of the thymus and lymph nodes with liberation of gamma globulins and immune bodies. I suspect, but have no proof, that if overproduction of steroids like Compound E was continuous, thymic involution would be accompanied by regenerative hyperplasia.† (If this were not the case, all the lymphoid tissue of the body would soon be exhausted).

\* However, it is easy to incorporate the fact in the adrenal theory. Theoretically, one hyperfunctioning cortex might depress the output of pituitary adrenotropin to such a degree that atrophy of the contralateral adrenal gland would result.

† Three months after this presentation was made we found regenerative hyperplasia at necropsy in one case. The patient died very shortly after admission to the clinic because of hypertension and heart failure. The only therapy employed consisted of the usual measures used in cases of congestive failure. The thymus was studied by Dr. Hal Downey.

Under such circumstances, carcinomatous changes in the thymus might be expected to occur, since carcinomatous changes seem to be particularly prone to develop in tissues in which breakdown and repair are accelerated. Several examples of this general phenomenon might be presented, but two will suffice. Gastric carcinoma not infrequently develops in the atrophic gastric mucosa of the patient who has pernicious anemia.<sup>38</sup> Carcinoma of the colon is a complication which occurs frequently in cases of ulcerative colitis.<sup>39</sup> In the case of Cushing's syndrome associated with carcinoma of the pancreas, one can only suspect that the pancreatic cells also may be in this state of flux. Irrespective of this point, there seems to be little doubt that the pancreas of the patient who has this syndrome is a particularly vulnerable organ, susceptible to cyst formation, abscess, fat necrosis, and carcinoma. Personally, I have seen examples of all these pancreatic complications. In a small series of cases reported by Mellgren,<sup>40</sup> although he did not call attention to them, there was a relatively high incidence of pancreatic complications.

Recently, Heinbecker<sup>41</sup> has described degenerative lesions in the paraventricular nuclei in cases of Cushing's disease without adrenal tumor. These changes, he concluded, were of pathogenic significance. He postulated that, when these nuclei degenerated, the pituitary body was released from hypothalamic control and that, as a consequence, the Crooke's cells\* would hyperfunction. This conception, it will be noted, is essentially a modification of the pituitary theory.

Finally, there remain a few disconcerting pathological observations that occasionally have been encountered. For example, Reforzo-Membrives described, in a personal communication, a case in which neither gross nor microscopic abnormalities could be discovered in either the pituitary body or the adrenal cortices. Crooke's changes, although carefully searched for, could not be found. Thompson and Eisenhardt<sup>12</sup> mentioned instances of Cushing's syndrome associated with chromophobe adenomas of the pituitary body. The case of Freyberg, in which a basophilic tumor of the pituitary body was associated with adrenal cortical hypoplasia, has been mentioned previously. For the time being, these and similar cases can best be regarded as inexplicable exceptions to the general rule.

### *Clinical Evidence*

In considering the etiology of Cushing's disease, one must keep in mind two major clinical observations.

First, a significant number of patients apparently have either been cured of their symptoms or materially benefited by roentgen therapy to the pituitary body. In my experience, the number of patients benefited by this type of treatment has not been impressive. Nevertheless, when remissions have followed roentgen therapy, they have been rather dramatic. Other clinicians, particularly Luft,<sup>13</sup> have reported more impressive results. It seems only fair, therefore, to accept the evidence at its face value and assume that the cure or remission was induced by the roentgen therapy that was em-

\* For brevity, I have used this term to designate pituitary basophilic cells which have the abnormal cytological appearance described by Crooke.

ployed. Failures conceivably might occur because a basophilic tumor was not present, or, if one was present, it was roentgenologically insensitive, or because in the presence of Crooke's changes the disordered cellular pathological physiology was not amenable to roentgen therapy. Ignoring the failures and concentrating on the successes, one is tempted to regard favorable response to roentgen therapy as proof for the pituitary etiology of Cushing's disease. Regarded as a problem in pure logic, it is relatively easy to show that this conclusion need not be correct. The erroneous reasoning arises from the fact that a distinction is not made between an etiologic factor and a necessary condition.\*

To make this point clear, I am going to consider for a moment the etiology of malaria as it appeared a century ago. It was known then not only that the presence of swamps or stagnant water, temperature, and direction of prevailing winds had an important influence on the incidence of malaria, but also that, when some of these factors changed, the epidemiological characteristics of the disease were altered. With the evidence available at the time, it was highly logical, but nevertheless completely erroneous, to conclude that malaria was caused by stagnant water, for example.

In a similar fashion, one can also regard the influence of the pituitary body in the pathogenesis of Cushing's syndrome as a condition necessary to its production rather than as a primary etiological factor. If this were the case and if the function of the pituitary body were altered by roentgen therapy, the syndrome might be expected to regress in some instances, just as the incidence of malaria might be greatly reduced when conditions necessary to the breeding of mosquitoes were eliminated. In short, then, favorable response to roentgen therapy alone does not necessarily establish the validity of the pituitary theory.

The second clinical observation of theoretical importance is the fact that Cushing's syndrome has been cured after the removal of an adrenal cortical tumor (FIGURES 1 and 2). This fact establishes the adrenal neoplasm as the immediate source of the endocrine symptoms. The evidence for this particular point is impressive. In one of the cases of Cushing's disease which I have studied, the patient at some time in the course of her illness had all of the symptoms mentioned by Cushing, as well as others, such as hypochloremic and hypokalemic alkalosis. Following removal of an adrenal cortical neoplasm, all of these symptoms disappeared. The tumor recurred and the symptoms did likewise. Removal of the recurrent tumor was followed by remission of symptoms. Finally, the recurrent tumor recurred and metastasized and once again the endocrine symptoms reappeared. Examples of this sort, however, do not prove that the disorder did not in reality begin as a specific type of basophilic dysfunction. In fact, a clinical course such as the one just described might be expected if the Crooke's cells (which, by the way, were present) initiated the disturbance and then continued to act after the tumor was removed. Cases of this type strongly suggest, but do not prove, that the hyperplastic adrenal cortices of Cushing's disease are

\* It is rather remarkable how infrequently this distinction has been considered in current endocrinological theories regarding the relationship of the pituitary body to the pathogenesis of various diseases, such as hyperthyroidism and diabetes mellitus.



FIGURE 1. Cushing's syndrome associated with adrenal cortical tumor; *a* and *b*, appearance of patient before operation.

the immediate cause of the symptoms. One might argue that hyperfunctioning basophilic cells *per se*, without the mediation of the adrenal cortices, could produce clinical phenomena that are essentially the same as those produced by hyperfunctioning adrenal cortical cells. In this connection, the case of Freyberg carries some weight. Furthermore, diabetes produced experimentally, either by the administration of anterior pituitary preparations or by adrenal steroids or extracts, is an example of a single biological effect brought about by the action of two entirely different agents. Intuitively, however, the supposition that a distinctive, complex, and unusual clinical picture might be brought about by either one of two entirely different mechanisms is distasteful. Moreover, in the case of the malignant adrenal tumor previously mentioned, if the Crooke's cells were the immediate cause of any of the endocrine symptoms, the remission that followed the removal of the tumor should have been incomplete, or should not have occurred at all.

In his Harvey Lecture, Albright<sup>20</sup> summarized clearly and succinctly what I have called the adrenal theory:

*Major Premises*

A. Patients with Cushing's Syndrome presumably have some common denominator in the etiology, as it would be unlikely that two entirely unrelated disorders would produce such a combination of clinical findings.

B. Patients with cancer of the adrenals and Cushing's Syndrome presumably are suffering from an hyperadrenocorticism of some kind; *ergo* all patients with Cushing's Syndrome are suffering from an hyperadrenocorticism.<sup>21</sup>



FIGURE 2. (Same patient as FIGURE 1.) Appearance of patient and her child four years after the removal of a benign encapsulated tumor (4 cm. in diameter, 9.0 gm. in weight) of the right adrenal gland. The left adrenal gland was examined also. Grossly, it appeared to be atrophic. A tiny portion was removed and this portion appeared to be atrophic on histologic examination. Eight years after operation the patient wrote that she had two children and considered herself to be well.

The logical consequences of the second of these two premises are far-reaching. I shall express them in a quasi-mathematical manner.

- A. Assume that the abnormal basophilic cells in the pituitary body (either the Crooke's cells or the basophilic adenomas) are responsible for the pathogenesis of Cushing's syndrome.
- B. Since none of the symptoms of the syndrome need be attributed directly to abnormal basophilic function, and since all of the symptoms can be attributed to adrenal cortical hyperfunction, then, the part played by the basophilic cells in the pathogenesis of the syndrome probably is indirect, remote, and restricted to the production of only one type of hormonal agent, presumably adrenotrophic in character. (If more than one basophilic agent were produced, endocrine symptoms would persist after the removal of a hyperfunctioning adrenal cortical lesion.)
- C. If the abnormal Crooke's basophilic cells produce only one type of

hormonal agent, normal basophilic cells *a priori* might produce only one type of hormonal agent.

- D. If normal basophilic cells produce only one type of hormonal agent, all other types of anterior pituitary hormonal agents are produced by the nonbasophilic cells.
- E. Indirect experimental, clinical, and pathological evidence strongly suggests that basophilic cells are not restricted to the production of one type of hormonal agent. Therefore, *D* becomes untenable, or at least unlikely.
- F. If *D* is fallacious, *A* is reduced, if not to an absurdity, at least to an improbability. In other words, neither the Crooke's cells nor the basophilic adenomatous cells play any part in the pathogenesis of the syndrome.

Another deduction follows. If the basophilic adenomas play no part in the pathogenesis of the syndrome, they may, like the Crooke's cells, be regarded as a retrograde phenomenon. If this were the case, a sequence of events such as the following might account for their presence. For some unknown reasons, the adrenal cortex begins to hyperfunction. The adrenal steroids discharged from the cortex have an adverse or even lethal action on the basophilic cells of the pituitary body, which then acquire the histologic appearance described by Crooke. As a compensatory phenomenon, adenomatous basophilic hyperplasia results, analogous to the nodular adenomatous-like hyperplasia which occurs when the liver is damaged experimentally by toxic agents.

It should be noted that the entire argument rests on a major premise which appears to be reasonably probable, but which has not been proved, namely: in all cases irrespective of the remote cause of the syndrome, the endocrine symptoms result from some sort of adrenal cortical hyperfunction. It should also be noted that I have disregarded one other possibility because, *a priori*, it seems unlikely, namely: in Cushing's syndrome for some unknown reason, pituitary basophilic hyperfunction is dissociated in a peculiar and specific fashion so that the abnormal cells make only one of several possible agents in excessive amounts. Eosinophilic tumors almost certainly produce several trophic agents. Reasoning by analogy, it seems unlikely that functioning basophilic tumors should be restricted to the production of only one trophic principle if the normal basophilic cells have polyhormonal potentialities.

### *Experimental Evidence*

The experimentalist has contributed comparatively little that bears on the immediate problems that pertain to the etiology of Cushing's syndrome. So far as is known, neither domestic nor experimental animals are subject to Cushing's syndrome, and, to my knowledge, in contrast to acromegaly and gigantism, nothing comparable to Cushing's syndrome has ever been produced experimentally. Most of the efforts of the experimentalists have been directed toward the study of the effects that follow the administration of specific pituitary principles or various adrenal steroids. In these studies,

isolated symptoms of the disorder have been produced, particularly if adrenal steroids were administered. From this type of investigation, it is possible to understand how many of the symptoms of Cushing's syndrome might be produced. For a detailed analysis of the mechanisms by which adrenal cortical hyperfunction might produce the symptoms I have mentioned earlier, I refer the reader to Kenyon's<sup>42</sup> review in "Surgery," Albright's Harvey Lecture,<sup>20</sup> and the manuscript which my associates and I prepared for the 1946 Laurentian Hormone Conference.<sup>43</sup> Very little of this experimental work, however, throws any light on the problem which I am discussing, namely: Is Cushing's syndrome a primary disorder of the adrenal cortices?

Two types of experimental study seem to bear directly on my subject. The first has to do with the experimental production of Crooke's changes. Creditable cytological changes comparable to those described by Crooke have been produced by Severinghaus and Thompson<sup>44</sup> by the administration of pituitary extracts. Here it is possible that the pituitary extract stimulated the adrenal cortex to hyperfunction and, by so doing, caused retrograde changes, of the type described by Crooke, in the basophilic cells of the pituitary body. The second pertains to the experimental production of anterior pituitary tumors. Chromophobe tumors of the anterior pituitary body have been produced experimentally by the administration of large amounts of estrogenic substances.<sup>45, 46</sup> If chromophobe tumors can be produced by steroids of this type, it is not unreasonable to think that in suitable subjects basophilic tumors might be induced by the administration of adrenal or other steroids.

### *Résumé of the Theories Regarding the Pathogenesis of Cushing's Syndrome*

In TABLE 1, I have attempted to outline the more important theories regarding the pathogenesis of Cushing's syndrome. None of these theories has been established conclusively, nor has any one of them been definitely refuted. For the time being, I regard theories 4 and 5 as being the most probable, because, to me, they conform with the greatest amount of factual material obtained from clinical, pathological, and experimental sources.

In my discussion thus far, I have used the words "theory" and "assumption" frequently. This was done deliberately, because to date every form of therapy that has been advocated has been based on some underlying theory or assumption or both. As long as this state of affairs continues, theories and assumptions will have more than an academic interest and importance.

My concluding remarks will be devoted to a description of the consequences that followed the practical application of one theory and to some of these consequences which appeared somewhat unexpectedly and proved to be of considerable scientific interest.

### *Recently Acquired Clinical Data\**

The lack of any specific medical treatment, the uncertain response to roentgen therapy to the pituitary body, and the miserable prognosis that confronts many of these patients led Dr. Walters and me a number of years ago to

\* This material was presented in September, 1946, at the Laurentian Hormone Conference.



TABLE 1  
THEORIES REGARDING THE PATHOGENESIS OF CUSHING'S SYNDROME

Theory	Pathological changes induced by unknown remote cause			Pathological changes involved in the immediate cause of symptoms			Associated pathological changes		
	Organ	Pathological change	Organ	Pathological Change	Organ	Changes	Explanation for		
1	Anterior pituitary body	Crooke's cells or basophilic adenoma or both	Anterior pituitary body	Crooke's cells or basophilic adenoma or both	None				
2	Anterior pituitary body	Crooke's cells or basophilic adenoma or both	Adrenal cortex	Hyperplasia or neoplasia	Adrenal cortex	Hyperplasia or neoplasia	Stimulated by pituitary basophilic hyperfunction		
3	Anterior pituitary body	Crooke's cells or basophilic adenoma or both	Adrenal cortex Anterior pituitary body	Hyperplasia Crooke's cells or basophilic adenoma or both	Adrenal cortex	Hyperplasia			
4	Adrenal cortex	Bilateral hyperplasia	Adrenal cortex	Bilateral hyperplasia	Anterior pituitary body	Crooke's cells	(1) Retrograde from adrenal cortex (2) Adenomatous regeneration		
5	Adrenal cortex	Unilateral neoplasm	Adrenal cortex	Adrenal cortical neoplasm	Anterior pituitary body Adrenal cortex	Crooke's cells Contralateral atrophy	Retrograde from adrenal cortex Mediated by anterior pituitary body		

attempt to treat a few patients with Cushing's disease surgically. Generally, less than half of each adrenal gland was removed. The therapeutic results were not impressive.

Subsequently, as it became apparent that the symptoms might well be an expression of adrenal cortical hyperfunction, my associates and I decided that if more adrenal tissue were removed the results might be better. For a time, we considered seriously the advisability of performing total adrenalectomy. Ultimately we decided, however, to resort to a less radical procedure, feeling that there was a reasonable chance that subtotal bilateral adrenalectomy might be equally as effective and at the same time be less hazardous to the patient.

In the three years prior to March, 1947, four patients were selected for this type of therapy. In three (cases 1, 2, and 4), all of one adrenal gland was removed and subsequently about two thirds of the other; in a fourth (case 3), about 60 per cent of each adrenal gland was removed.

The postoperative course in each instance was almost identical in character, and I shall describe it here in considerable detail. Two digressions are indicated.

The first digression concerns the patients. All four, of whom three were women and one was a man, had the classic Cushing's syndrome in its worst form. One patient, the man (case 1), had congestive heart failure and was edematous to the waist line. All had severe hypertension. In only one of the cases was the urinary excretion of 17-ketosteroids significantly increased. One patient (case 3), at the second operation, was found unexpectedly to have a low-grade adrenal carcinoma about 1 inch (2.5 cm.) in diameter. We have reasons to believe that this carcinoma may not have been hyperfunctioning.

The second digression has to do with the ordinary sequence of events which occurs when a hyperfunctioning adrenal tumor is removed on one side and the opposite gland is either atrophic or functionally inadequate. If the patient has not been properly prepared, there develops, during the first twenty-four hours or shortly thereafter, because of transient, contra-adrenal cortical atrophy, a shock-like condition which is often fatal. The temperature rises to hyperpyrexial levels and the clinical picture resembles fulminating adrenal cortical insufficiency. If, by virtue of treatment, the patient does not die, improvement generally proceeds rapidly, and at the end of a week or more, it is obvious that he will recover. Three months after the operation, many of the symptoms will have disappeared, and ultimately, provided that there has been no recurrence of the tumor, the diagnostic signs of the former malady will have regressed completely. If all adrenal cortical tissue is removed, as seemed to be true in the case of Bartels<sup>17</sup> of the Lahey Clinic, in which a nonfunctioning tumor was removed, presumably arising from a solitary adrenal gland, ordinary Addison's disease seems to follow. In his case, immediately after the operation the classic picture of postoperative adrenal insufficiency developed. With treatment the patient, a woman, recovered. When treatment was reduced or discontinued, adrenal insufficiency recurred. Soon, it became apparent that continuous replacement therapy would be necessary. At the end of three months she was deeply

pigmented and had all the other characteristic signs and symptoms of Addison's disease.

Let us return then to the postoperative course in the four cases under discussion. With these an entirely different sequence of events occurred, a picture of which we shall try to present in a composite form. Immediately after partial removal of the second adrenal gland, nothing of any particular moment happened. Convalescence seemed to proceed uneventfully. Some time during the first week after operation or shortly thereafter, the patients complained of nausea. They lost their appetite and vomited. The anorexia became more and more intense, until finally they refused almost all food. Even the sight of food became repulsive. About the same time, abdominal pain and tenderness made their appearance. Gradually, the chemical composition of the blood became disordered. This disturbance generally was characterized, when the patient was not being treated with adrenocortical extract or electrolytes, by low concentrations of both sodium and potassium. The values for blood urea, and, as time went on, the concentration of blood calcium, slowly tended to rise and eventually attained levels seen in hyperparathyroidism. Phosphate levels, however, were not depressed.

Little by little, the patients lost their "Cushingoid" appearance. The plethora faded and the skin became pallid. Striations disappeared, ecchymoses no longer occurred after venipuncture, and it became possible to take the blood pressure without the production of petechiae. Imperceptibly, the hirsutism of the women vanished, so that the extremities became almost hairless. Growth of axillary hair was retarded. In every case, the urinary excretion of 17-ketosteroids declined until it reached levels characteristic of Addison's disease. The blood pressure varied but, in each instance, hypertensive values were obtained on most days irrespective of treatment. In case 3, administration of sodium chloride and sodium bicarbonate was followed by a sharp rise in blood pressure. Differential blood counts disclosed a gradual increase of the percentage of lymphocytes.

One of the patients, the man in case 1, seemingly was cured. Prior to the operations, his condition was the worst of the group. Two months after his second operation he was able to leave the hospital. By this time his condition was beginning to improve. During the next nine months he was seen on two occasions and, at each visit, fairly complete examinations did not reveal abnormalities of any consequence (FIGURE 3). His blood pressure had returned to normal and there was no question about the fact that he had neither Cushing's syndrome nor Addison's disease. The chemical composition of the blood and the daily urinary excretion of ketosteroids (10.7 mg.) were normal. Libido and potency, which had completely disappeared, had returned. At his last examination, eleven months after his second operation, his only complaint was that his physical stamina were below par as measured by his former standards. Nevertheless, he fathered a child. Some data in this case are summarized in TABLE 2.

Two of the remaining three patients (cases 2 and 4) died after a protracted illness (forty-two and sixty-six days). Both had gross disease of the pancreas. In one (case 4), there was evidence of acute and chronic



FIGURE 3. Cushing's syndrome not associated with adrenal cortical tumor: *a*, appearance of patient before bilateral subtotal adrenalectomy; *b*, appearance of patient 6 months later; *c*, appearance of patient about one year after operation.

hemorrhagic pancreatitis with cyst formation. This condition may have been present before her first operation, if any conclusions can be drawn from the medical history. In the other patient (case 2), fat necrosis was found in the tail of the pancreas. All of this is reminiscent of the "beef steak" pancreatitis that was described by Mann and Drips,<sup>48</sup> and at a later date by Code,<sup>49</sup> following adrenalectomy in dogs. Code has told me that these dogs refuse to eat and usually die, but, if they are fed by tube, some of them recover. Data in case 2 are given in TABLE 3.

In one patient (case 4) at necropsy, no adrenal cortical tissue could be identified. A basophilic adenoma about 2 mm. in diameter was found in the pituitary body, and, in the nonadenomatous portion of the gland, Crooke's changes were present in approximately 40 per cent of the basophilic cells. In the case of the other patient who died (case 2), traces of adrenal cortical tissue were identified. Unfortunately the pituitary body could not be examined.

The fourth patient (case 3) is alive four months (March, 1947) after removal of most of her adrenal cortical tissue and she is slowly improving. She no longer has any of the features of Cushing's syndrome that can be recognized on inspection. On the other hand, she does not have the appearance

TABLE 2  
COURSE AFTER SUBTOTAL ADRENALECTOMY: CUSHING'S DISEASE—ADRENAL HYPERPLASIA (CASE 1, MAN, AGED TWENTY-NINE YEARS)

Date	Plasma					Blood pressure  <i>mm. Hg</i>	Antecedent treatment				17-keto-steroids	Lympho- cytes		
	mEq. per liter			Mg. per 100 cc.			Electrolyte		Hormonal					
	Na	Cl	CO <sub>2</sub>	K	Ca		P	NaCl	Na citrate	KCl			Cortical extract	Testos- terone
7-17-45	139	99	34.4	4.0	10.4	3.5							<i>mg. in 24 hr.</i>	%
8-7-45	Left adrenalectomy						175/135							
8-28-45	136	100	28.0	4.7			150/100					+	30.9	12
9-7-45	Right subtotal adrenalectomy						145/85						15.6	
9-26-45	140	96	34.1	4.7			170/110					+		
10-2-45		101	30.0		13.9		148/90							
10-9-45	144	101	38.0	3.1	14.1	3.76	160/100					+		
11-2-45	135	101	30.0	3.3	13.7	3.36	160/120	+	+			+	3.3	
	Remission						130/80							
2-7-46	136	101	29.0	4.5	10.2	3.5	134/90						8.6	
8-6-46	136	100	26.0	5.4	10.0	4.1	132/90						10.7	21

TABLE 3  
COURSE AFTER SUBTOTAL ADRENALECTOMY: CUSHING'S DISEASE—ADRENAL HYPERPLASIA (CASE 2, WOMAN, AGED TWENTY-NINE YEARS)

Date	Plasma						Blood pressure  <i>mm. Hg</i>	Antecedent treatment		17-keto-steroids  <i>mg. in 24 hr.</i>	Lympho- cytes  %
	mEq. per liter			Mg. per 100 cc.				Electro- lyte	Cortical extract		
	Na	Cl	CO <sub>2</sub>	K	Ca	P					
11-13-45	135	103	26	4.2	9.1	3.3	170/110			1.2	6
11-23-45	Left adrenalectomy						165/125		+	2.9	
11-30-45	136	100	28	4.5	7.5	3.6	160/115				
3-2-46	Right subtotal adrenalectomy						150/100				10
4-2-46	135	93	31		11.8	2.9	168/145	+	+	1.4	
4-8-46				4.9	13.4	2.8	190/140	+	+	0.3	
4-14-46	134	99	25.2				170/110	+	+		29
5-1-46	136	100	23	2.6	10.3		160/120				
5-7-46	137	110		5.0	8.9	4.4		+	+		

of patients who have Addison's disease. There is no pigmentation of the skin and repeated attempts to induce pigmentation by exposure to ultraviolet light have failed. The skin neither burned nor tanned. Comparatively early in her postoperative course, she complained of severe, continuous, upper abdominal pain. This finally disappeared after treatment with streptomycin was instituted, and, at the time, we suspected that she also had some type of pancreatitis. Her outstanding residual symptoms have been anorexia and profound muscular weakness. She still has mild hypertension and there is evidence of impaired renal function, although both of these seem to be improving slowly (TABLE 4).

In all four instances, it was difficult to make a distinction between those symptoms that were part and parcel of the postoperative disorder itself and those that might have been induced by treatment, either hormonal or with electrolytes. In the face of a decreased concentration of sodium in the plasma, we naturally were reluctant to discontinue the administration of cortical extract and sodium chloride, and, as a consequence, all four patients received such treatment in liberal amounts for varying periods after operation. In one patient (case 3), who had undergone partial removal of both adrenals, however, we decided to take the risk and discontinue all treatment. This was done for a period of about one month. During this month there was no appreciable change in the patient's clinical condition. The general level of the blood pressure fell but still persisted at levels that could be regarded as hypertensive. Azotemia persisted. The concentration of sodium in the blood remained low, while that of potassium slowly increased and finally reached low normal values. The excretion of 17-ketosteroids in the urine remained at Addisonian levels.

At the end of this period of study, testosterone propionate was administered in doses of 25 mg. three times a week. The patient's general condition immediately began to improve. Her appetite picked up, she became stronger, and the waxy pallor of the skin was replaced by a faint rubor. Thus far, this treatment has had no effect on the level of the blood urea or the blood pressure. Values obtained in the last chemical analysis the blood were as follows: sodium 135, mEq.; potassium, 4 mEq.; chloride, 104 mEq.; bicarbonate, 27 mEq. (all per liter); calcium, 12.4 mg.; phosphorus, 3.5 mg.; and urea, 80 mg. (all per 100 cc.).

From these four cases, the following deductions seem to be justified: (1) Most of the symptoms of Cushing's syndrome are contingent on the presence of the adrenal cortices. (2) It is equally clear that in the absence of the adrenal cortices most of the symptoms of Cushing's syndrome are not contingent either on the presence of a basophilic adenoma or of Crooke's changes. (3) The persistence of Crooke's changes in one case, in the absence of all demonstrable adrenal cortical tissue, casts some doubt on the thesis that Crooke's changes are manifestations of a retrograde or inhibitory action of the adrenal cortices on the anterior pituitary body. However, Crooke's changes may possibly be permanent changes. Furthermore, sufficient time (sixty-six days) may not have elapsed following removal of adrenal cortical tissue for Crooke's changes to disappear. (4) The

TABLE 4  
COURSE AFTER SUBTOTAL ADRENALECTOMY: CUSHING'S DISEASE—ADRENAL HYPERPLASIA (CASE 3, WOMAN, AGED THIRTY-EIGHT YEARS)

Date, 1946	Plasma					Blood pressure	Antecedent treatment			17-keto- steroids	Lympho- cytes		
	mEq. per liter			Mg. per 100 cc.			Electrolyte					Cortical extract	
	Na	Cl	CO <sub>2</sub>	K	Ca		P	NaCl	Na citrate				KCl
4-26	142	103	33	4.5	9.4	2.5					mg. in 24 hr.	%	
5-8	Partial left adrenalectomy												
5-11	137	96		4.3							10.0	16	
5-22	Partial right adrenalectomy, removal of cortical tumor												
5-27	138	100	—	2.7	8.9	3.2					7.5		
6-3	134	100	—	6.0	9.8	4.8				+	1.8		
6-10	131	95		3.8							0.6	21	
6-26	129	92		4.9	10.8	4.6					0.8	34	
7-8	135	100	26	3.4							0.7		
7-15	135	93	27	3.4							0.4	35	
7-26	130	91	29	3.9	13.9	4.5				+	0.2	45	
8-19	135	104	27	4.1	12.4	3.5					0.5		



hypertension of Cushing's syndrome does not depend entirely on the presence of the adrenal cortices. The adrenal cortices may be necessary for the original occurrence of this hypertension, but they do not seem to be necessary for the continuation after it once has been initiated. (5) In cases of Cushing's syndrome, removal of most of the adrenal cortical tissue may be followed by an unusual or at least atypical form of adrenal cortical insufficiency, the manifestations of which are different in many respects from those which are seen in cases of Addison's disease. (6) Subtotal adrenalectomy seems to be a hazardous procedure in cases of Cushing's syndrome. Its therapeutic utility has not been established, in spite of the fact that one patient seemingly has been cured of his disorder and another is getting better.

To return to the question implied in my title—"Is Cushing's disease a primary disorder of the adrenal cortex?"—the answer at this time is that I do not know; but it might well be.

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## EXPERIMENTAL ANAPHYLAXIS IN LOWER ANIMALS

By Beatrice Carrier Seegal

*Department of Bacteriology, College of Physicians and Surgeons, Columbia University,  
New York*

Anaphylaxis is a state of induced, specific hypersensitivity. Between 1898 and 1902, Richet<sup>1</sup> introduced the name and initiated the experimental study of this phenomenon. A wealth of information, helpful in the understanding of allergic diseases as well as anaphylaxis, has been accumulated. It is the purpose of this paper to present some of these data, especially those peculiarly pertinent to a monograph on allergy.

The demonstration of anaphylaxis is influenced by a number of variables. Among these are the antigen used for sensitization, the species of animal under investigation, and the method employed to elicit the hypersensitive response. There are three ways in which the hypersensitive response is demonstrable: as anaphylactic shock, as a local inflammatory reaction, or as a widespread inflammatory reaction. The state of anaphylaxis may be achieved passively. When a normal animal is injected with the blood of an anaphylactic animal the recipient also becomes anaphylactic.

*Antigen.* Different antigens require different quantities for sensitization. Crystalline egg albumin or whole horse serum, when injected parenterally in amounts containing only a few  $\mu\text{g.}$ , will sensitize the guinea pig so that fatal anaphylactic shock follows the subsequent injection of antigen.<sup>2, 3, 4</sup> Crystalline bovine albumin<sup>5</sup> or crystalline tobacco mosaic virus<sup>6</sup> similarly injected require several milligrams to sensitize and even then fatal shock often does not follow the challenging injection of antigen. When simple chemical substances such as picryl chloride or 2:4 dinitrochlorobenzene are injected repeatedly, intracutaneously in guinea pigs in amounts of 2.5  $\mu\text{g.}$  per injection, typical symptoms of anaphylaxis, and often anaphylactic death, follow the intravenous injection of the specific chemical coupled to a protein.<sup>7</sup> In this latter case it is impossible to know just how much of the injected chemical was responsible for sensitization, since the sensitizing power of such simple chemical substances is probably due to their capacity to combine with protein in the host to form complete antigens.

The physical state in which antigen is administered influences the quantity needed to induce maximum sensitivity. For example, if bovine albumin is precipitated with aluminum hydroxide and this alum precipitate is used to sensitize, the antigen is much more anaphylactogenic than when in solution. A single injection of  $\frac{1}{2}$  mg. will render all guinea pigs maximally sensitive.<sup>8</sup> Crystalline ovalbumin, when alum-precipitated, is 4 to nearly 400 times more efficient, depending on the route of administration.<sup>4</sup>

The route by which antigen is given has much to do with the quantity needed to sensitize. Coulson and Stevens<sup>4</sup> present most useful quantitative data. They find, for example, the subcutaneous superior to the intraperitoneal or intravenous route for sensitization of the guinea pig with alum-precipitated ovalbumin. By the former route as little as 0.04  $\mu\text{g.}$  served to sensitize. More than twice this amount was required if the sensitizing

injection was given intraperitoneally, and a sixty-fold increase was necessary for intravenous sensitization. Guinea pigs may be sensitized by routes to which man is exposed, namely, by inhalation or ingestion of antigen.<sup>8</sup> In these circumstances large amounts of antigen are sprayed into the air which the animals breath or are added to their diet.

Doerr and Berger<sup>9</sup> have reported that the incubation period required for sensitivity to develop varies with the antigen. They observed that horse serum albumin required a longer incubation period than the euglobulin fraction. When very small amounts of antigen, of the order of 0.008 mg., were used to sensitize, the incubation period was prolonged for each antigen. An incubation period of 3 or 4 weeks has been used in the recent work<sup>4</sup> designed to determine minimal sensitizing dosages of ovalbumin. However, in the guinea pig at least, the state of sensitivity may persist for months or years.<sup>3</sup>

The quantity of antigen needed to release anaphylactic shock has usually been greater than that required for sensitization. The recent studies of Coulson and Stevens<sup>4</sup> have indicated that this is not necessarily the case. They report on a series of guinea pigs sensitized intra-abdominally with 100  $\mu$ g. of ovalbumin which were fatally shocked three weeks later by the intravenous injection of 6  $\mu$ g. of the ovalbumin.

The information so far cited concerning the factor of antigen in sensitization and shock has been obtained from studies in the guinea pig. Other animals are less susceptible to fatal shock and have not been extensively used for experiments in anaphylaxis. Grove<sup>10</sup> has reported studies in the rabbit. She found a series of subcutaneous, intraperitoneal, and intravenous injections of egg white resulted in a high degree of sensitivity in about 75 per cent of the animals. Similarly repeated injections of antigen are usually employed to sensitize other animals, such as the dog and mouse. Rats are peculiarly refractory to anaphylaxis. A table listing methods which have been employed in the sensitization of a number of animal species and the quantity of antigen used to shock is given in Gay.<sup>3</sup>

*Anaphylactic Shock.* The manifestations of shock vary with the species of animal. It may be recalled that anaphylactic death occurs within 3 to 5 minutes in the guinea pig and is preceded by slow, labored, gasping respirations, cyanosis, prostration, and convulsions. In the rabbit, the cyanosis, prostration, and convulsions occur but the respiratory difficulty is not present. When death ensues, it is also only a matter of minutes. The picture of anaphylactic shock in the dog is that of profound and prolonged prostration, associated with vomiting and bloody diarrhea. Death may be delayed for 1 to 2 hours or slow recovery may take place. Despite the variation in the appearance of the reaction, more careful analysis demonstrates that the symptoms are largely referable to two phenomena, contraction of smooth muscle and an increase in capillary permeability. The location of the smooth muscle, which shows maximum contraction, differs from species to species and hence the picture of shock varies. Increased capillary permeability is more evident in the dog than in the rabbit or guinea pig.

When the guinea pig dead of anaphylaxis is examined at autopsy, the lungs are found fully distended. Microscopic examination of the lungs

provides the explanation. The bronchioles are constricted and the mucous membranes lie in folds, further obstructing the lumen. Inspiration sucks air past this barrier into the alveoli, but the air remains trapped, unable to pass the obstruction upon passive expiration. By the use of radioactive iodine attached to ovalbumin, Dixon and Warren<sup>11</sup> have shown that the antigen in a shocked animal is found in more than twice the expected amount in the edematous, collagenous tissue of the bronchial wall and the adventitia of the pulmonary vessels. Autopsy of the rabbit following anaphylactic death reveals a right auricle and ventricle which are markedly distended, while the inferior vena cava and liver are engorged. In this animal, as shown by Coca,<sup>12</sup> the pulmonary arterioles constrict. The pulmonary blood pressure rises while the systemic blood pressure falls,<sup>13</sup> so that anaphylactic death in this animal also is due to asphyxiation because insufficient blood reaches the lungs.

In the dog, the smooth muscle of the small intestine responds most violently during anaphylactic shock.<sup>14</sup> There is also a marked increase in capillary permeability, as shown by edema and hemorrhage in the intestinal mucosa. The liver becomes greatly engorged with blood, a condition which also is probably referable to increased capillary permeability.<sup>15, 16</sup>

Smooth muscle from a sensitized animal, when exposed to antigen *in vitro*, contracts. This phenomenon, the Schultz-Dale reaction,<sup>3</sup> has been shown for both the small intestine and the uterus of the sensitized guinea pig. Grove<sup>17</sup> has demonstrated similar contraction of arterial smooth muscle from this animal. Sollmann and Gilbert<sup>18, 18a</sup> have studied the contraction of the bronchioles and the pulmonary arterioles of the rabbit lung. Thin sections of lung from sensitized animals, placed in Ringer-Locke solution and observed under the microscope, show visible contraction of both tissues upon the addition of the specific antigen.

A knowledge of the relation of anaphylaxis to antibody titer is of fundamental importance to the understanding of the mechanism of anaphylactic shock. The guinea pig may be fatally shocked in the absence of circulating antibodies, demonstrable by any *in vitro* test. Kabat and his associates,<sup>2</sup> during their studies of passive anaphylaxis, have shown the reason for this. The guinea pig may be *passively* sensitized with such a small amount of antibody that its presence cannot be demonstrated except by the highly sensitive biological method of anaphylactic shock. This work will be described later. Jackson<sup>19</sup> has attempted to determine the quantity of circulating antibody which is required to insure anaphylactic shock in the actively sensitized rabbit. She found that a high titer of circulating antibody does not guarantee death from anaphylaxis in the rabbit. However, all animals which succumbed to anaphylactic shock had at least 3.56 mg. of antibody protein per cc. of serum.

**Local Inflammatory Reaction.** When the test for anaphylactic sensitivity is made by the local injection of the specific antigen, a quite different type of hypersensitive reaction is obtained. The first experiments of this kind were those of Arthus, who gave rabbits repeated subcutaneous injections of horse serum. No reaction resulted following the first few injections. The



later injections, however, induced erythema and edema which developed over a period of hours. In some cases the local inflammatory response was so severe that necrosis and sloughing occurred in the course of a day or two. Microscopically the reaction begins almost immediately. The change in the status of the capillary wall has been subjected to direct observation by Abell and Schenck.<sup>20</sup> They followed the result of introducing horse serum into the moat of an ear chamber in rabbits sensitive to this antigen. Contraction of arterioles, slowing of the circulation, migration of leucocytes through the vessel wall, and clumping of the red cells were recorded. Gerlach<sup>21</sup> has compared the histological picture of the reaction in several species of animals and in man at varying time intervals following the injection of the antigen. The histopathology of this reaction is not distinctive from that of acute inflammation due to other causes.<sup>22</sup>

In the sensitized rabbit, the Arthus reaction may be elicited in all tissues so far investigated. If the reaction takes place in a vital organ, physiological disturbances result. For example, inhalation of an antigen by a sensitive animal may produce congestion of the lungs with the histological appearance of acute pneumonia.<sup>23</sup> The introduction of specific antigen into the pericardial cavity results in an acute pericarditis and myocarditis, which may be so severe as to cause death from cardiac failure.<sup>24</sup> Similarly, the introduction of antigen into the brain of a hypersensitive animal produces profound inflammation that is manifested by neuromuscular disturbances.<sup>25</sup>

It is possible to reverse the usual order in demonstrating local hypersensitivity, namely, to induce local sensitization by local application of the antigen and subsequently to elicit evidence of this by intravenous administration of the antigen. Such a reaction has been carried out in the rabbit eye.<sup>26</sup> If a foreign protein is placed in the anterior chamber of the rabbit eye, after two to three weeks the intravenous injection of the same antigen results in hyperemia of the iris and conjunctiva, edema of the conjunctiva, and laceration, all limited to the previously sensitized eye. This reaction develops about two hours following the shocking injection, reaches a maximum in five hours, and usually subsides within 24 hours. Parenteral injection of antigen is not necessary to elicit the reaction, since it was obtained in 11 of 38 animals in which the shocking dose of antigen was given in large amounts by stomach tube.

Such a locally sensitized eye may be kept continuously inflamed over a period of days if several antigens have been injected simultaneously into the anterior chamber for sensitization. Some weeks later each antigen in turn is injected intravenously on succeeding days. The inflammatory response may be obtained to each successive antigen.<sup>26</sup>

Desensitization follows the injection of the specific antigen, but is temporary. The eye may become inflamed upon subsequent tests with the same antigen after a suitable period of time. The mechanism for this return of sensitization may perhaps be found in a series of experiments designed to test the effect of non-specific inflammation upon the capacity of the eye to become sensitive to a circulating antigen.<sup>27</sup> A series of rabbits was divided into three groups: group one was injected with glycerin into

the anterior chamber of the right eye and, during the period of inflammation which followed, egg white was injected intravenously; group two received only the glycerin in the eye; and group three only the egg white, intravenously. Four weeks later all animals were injected intravenously with egg white. There was no reaction in groups two or three to this treatment, but in 12 of 21 animals belonging to group one a typical local inflammatory reaction in the glycerin-treated eye occurred. This experiment is interpreted to indicate that the circulating antigen is localized in the non-specifically inflamed eye and here serves to produce an area of local sensitization, which is demonstrated after a suitable incubation period by the intravenous injection of the specific allergen. An allergic inflammation should be highly efficacious for concentrating antigen, since the antigen is drawn to the area, first, by the specific antibody and, secondly, by the inflammation. Thus, new antigen is available for renewal of sensitization of the local area.

The eye is not the only organ which has been locally sensitized. It has been found that the rabbit heart may similarly exhibit local sensitization after antigen is injected directly into the pericardial cavity.<sup>28</sup> Rabbits were injected with 1 cc. of egg white into the pericardial cavity, while controls received the same antigen intraperitoneally or intravenously. Four to 7 weeks after the sensitizing injection the animals were sacrificed and heart preparations from each group were prepared according to the method of Wilcox and Andrus.<sup>29</sup> These organs were perfused with Ringer-Locke solution and the effect upon coronary flow of the addition of egg white was measured graphically. Fourteen of the 15 hearts from animals sensitized intrapericardially showed the typical anaphylactic reaction characterized by a drop of 22 to 64 per cent in the rate of flow of the perfusate through the coronary vessels. Only 7 of the 31 hearts taken from the other animals sensitized intraperitoneally or intravenously gave a positive reaction by showing a decrease in rate of coronary flow of 15 to 45 per cent. In short, the direct exposure of the heart to 1 cc. of antigen sensitized it, whereas a similar amount of antigen injected elsewhere was usually insufficient to sensitize the heart.

*General Inflammatory Response.* The third group of reactions which we are considering under experimental anaphylaxis are those which follow a single, large intravenous injection, or closely spaced multiple injections, of a foreign protein. "Serum sickness" has been produced experimentally in rabbits by Fleisher and Jones.<sup>30</sup> In these animals it is characterized by erythema and edema of the ears, which develops 3 to 8 days following intravenous injection of 5-10 cc. per kilogram of horse serum. Khorazo<sup>31</sup> observed the reaction following the injection of horse serum but failed to obtain the reaction when human, sheep, guinea pig, or dog serum was employed. We<sup>6</sup> have observed one typical serum sickness reaction in the ears of a rabbit 44 days following the intravenous injection of 1 gm. of crystalline bovine albumin. Three other animals had unilateral reactions which occurred in 20 to 30 days in the ear receiving the intravenous injection.

Rich has again focused attention on perivascular inflammation which can be found in rabbits following massive injections of antigen. Klinge<sup>32</sup>

described such lesions and has reviewed the earlier work. Rich and Gregory<sup>38</sup> have produced diffuse periarteritis nodosa by the same technique used to induce serum sickness in rabbits. A single large injection of horse serum given intravenously may result in about three weeks in widely scattered periarthral collections of leucocytes, which may invade to the intima and which are associated with fibrinoid and hyaline alterations and necrosis of the walls, producing the histopathological picture of periarteritis nodosa. These workers<sup>34</sup> also found cardiac lesions which, in their basic characteristics, resemble closely those of rheumatic carditis.

More recent observations of this type of anaphylactic reaction are those of Hawn and Janeway,<sup>35</sup> who worked with two single antigens, crystalline bovine albumin and a highly purified bovine gamma globulin. These two proteins derived from the same animal species gave rise to somewhat different diseases when injected intravenously in rabbits. The albumin disappeared more slowly from the circulation and appeared to be a poor antigen, but in those animals in which lesions were found, after a period of two to three weeks, they were distributed throughout the arterial system and mimicked those of periarteritis nodosa. On the other hand, the rabbits injected with gamma globulin developed lesions more rapidly and lost the foreign protein from the circulation more rapidly than in the case of rabbits given the albumin. The lesions, instead of being distributed throughout the arterial system, were predominantly in the myocardium and glomeruli of the kidney.

Ehrlich, Seifter, and Forman<sup>36</sup> have examined the pathologic changes resulting from the injection of varying amounts of horse or duck serum in rabbits which were sacrificed 3 to 34 days later. Allergic arteritis, marked glomerulonephritis, myocardial necrosis, and Aschoff-like bodies in the myocardium are described. Moore and his associates<sup>37</sup> have been successful in producing acute rheumatic-like heart lesions in mice by the repeated parenteral injection of egg white. More *et al.*<sup>38, 39</sup> have utilized bovine serum gamma globulin as well as horse serum in their studies on allergic serum disease. The horse serum produced diffuse arteritis and the bovine gamma globulin both glomerulonephritis and granulomatous lesions of the heart valves as well as an arteritis. A useful review of the literature is to be found in reference 38.

No account of the diffuse inflammatory reactions of hypersensitivity should fail to include mention of the lesions which have been produced by the injection of homologous and autogenous antigens. The Caveltis<sup>40</sup> have reported that the injection of rats with homologous rat kidney, mixed with killed ( $\delta$ ) hemolytic streptococci, produces a chronic progressive nephritis in these animals. Kabat, Wolf, and Bezer<sup>41</sup> and Morgan<sup>42</sup> have produced an acute disseminated encephalomyelitis, resembling multiple sclerosis, by injecting monkeys intramuscularly with heterologous or homologous suspensions of brain or spinal cord. These antigens were mixed with killed tubercle bacilli and suspended in oil previous to injection, according to the adjuvant technique of Freund and McDermott,<sup>43</sup> which serves to enhance antibody production. Kabat has shown that injection of a monkey with a portion of

its own cortex, removed surgically, serves as well to induce the lesions.<sup>44</sup> Hydrolized, autogenous rabbit serum or saline extract of the rabbit's skin have been used by Gosset, Jahiel, and Delaunays<sup>45</sup> and Jahiel and Jahiel<sup>46</sup> to sensitize the rabbit's own lung. A month after the initial injection of 0.5 cc. into the lung parenchyma an intravenous injection of the same material produces an acute pneumonitis of the previously sensitized lung.

*Passive Sensitization.* The serum of an anaphylactic animal may be capable of passively sensitizing a normal animal. This was first demonstrated by injecting a normal guinea pig with the serum of a sensitized guinea pig.<sup>3</sup> Four hours later the injection of the specific antigen elicited anaphylactic shock. Antibody-containing serums from the rabbit, as well as from the goat and man, have also been found capable of passively sensitizing the guinea pig to anaphylactic shock, whereas antisera from the rat, the horse, the chicken and from cattle have failed to accomplish this passive sensitization. On the other hand, horse antipneumococcus serum has passively sensitized the guinea pig so that an immediate wheal and erythema skin reaction,<sup>46</sup> or an Arthus-like reaction,<sup>47</sup> follows the intracutaneous injection of the carbohydrate antigen. Likewise, horse antipneumococcus serum transfers the Arthus type of hypersensitivity to the rabbit<sup>48</sup> and the anaphylactic type of hypersensitivity to the dog.<sup>49</sup> It is thus apparent that passive transfer of hypersensitivity is conditioned by the donor and recipient hosts and by the nature of the test employed to demonstrate the hypersensitive state.

Quantitative studies in passive sensitization of the guinea pig and rabbit, utilizing sera of chemically defined antibody titer, have yielded most interesting data concerning the amount of antibody necessary to accomplish sensitization. Kabat and his associates<sup>50</sup> have shown that only 0.03 mg. of anti-crystalline egg albumin antibody N or antipneumococcus type III antibody N is sufficient, when injected intravenously, to render a 250 gm. guinea pig susceptible to lethal anaphylactic shock, when challenged with the specific antigen 48 hours later. Three one-hundredths of a milligram of antibody nitrogen, when distributed throughout the circulation and body tissues, is not detectable by *in vitro* methods. Indeed, when five times this amount of anti-egg albumin N was injected it still could not be demonstrated 48 hours following injection. This indicates that the biological test of anaphylaxis is better able to detect antibody than the presently available test-tube tests. Another striking illustration of this is furnished by the observation that the excised uterus from a guinea pig sensitized passively with 0.03 mg. of anti-egg albumin N will show the typical Schultz-Dale reaction. The addition of 1 mg. of antigen to the bath containing the uterine strip causes contraction. Kabat and Landow<sup>51</sup> have calculated that the uterus probably contains amounts of antibody N of the order of 0.01  $\mu$ g.

Fischel and Kabat<sup>52</sup> and Benacerraf and Kabat<sup>47</sup> have obtained data on the quantitative relation between antigen and antibody in the passively induced Arthus reaction. As in anaphylactic shock, small quantities of antibody sufficed to induce sensitivity. If the sensitizing injection was

given intracutaneously, 0.025 mg. of antibody N sensitized the rabbit locally, and 0.09 mg. the guinea pig.

The quantitative studies on anaphylaxis in Kabat's laboratory have disclosed a very interesting role which the nature of the antibody plays in passively induced anaphylactic shock and the Arthus reaction. If non-precipitable or "univalent" rabbit antiovalbumin was utilized passively to sensitize the guinea pig to anaphylactic shock, 0.03 mg. of this antibody was required. The non-precipitating antibody was as efficient as the precipitating antibody.<sup>53</sup> On the contrary, equivalent weights of nonprecipitable antibody were unable to prepare the animals for a passive Arthus reaction.<sup>47</sup>

The time which must elapse between the injection of antibody and the development of hypersensitivity in the passively sensitized animal is a factor both of the animal species and of the quantity of antigen. The rabbit becomes sensitive immediately after injection of the homologous antiserum,<sup>54</sup> as do the dog and mouse. In the rabbit, passive sensitivity is rapidly lost, but may last 20 days in the dog.<sup>15</sup> It has been concluded from most of the observations in guinea pigs that an incubation period of two hours or more following the injection of the antiserum is necessary to accomplish passive sensitization. This statement is at variance with some of the reported data.<sup>16</sup> Benacerraf and Kabat<sup>55</sup> have carried out quantitative studies concerning the relation of the amount of sensitizing antibody to the minimum latent period before anaphylactic shock is demonstrable. When the sensitizing dose of 0.03 mg. antiovalbumin N is increased approximately 30 times, animals may be fatally shocked when antigen is injected immediately after the antiserum. A fourteen-fold increase in antibody resulted in fatal shock one-half hour later. As these authors state, a reaction of some kind takes place as the interval between sensitization and shock is lengthened, which increases the efficiency of a given quantity of antibody in effecting sensitization.

*Discussion.* Allergic diseases of man were among the diseases of unknown etiology until experimental observations in animals discovered the methods to establish the state of anaphylaxis or hypersensitivity. The fact that *repeated* exposure of an animal to an *initially* harmless substance might induce a serious or even fatal reaction was then found to have its counterpart in human experience. Once it was accepted that acquired hypersensitivity to agents in our environment might cause a variety of diseases, such as hay fever, asthma, or urticaria, the list of possible allergic diseases of man grew rapidly. Soon the many manifestations of known allergic diseases and of suspected allergic diseases outstripped the experimentally induced hypersensitivities. Because many manifestations of allergic diseases in man failed to find a suitable counterpart in experimental work in animals, the question has arisen in the minds of many as to whether certain of the allergic diseases of man are unique to him. It would seem, as further experimental work develops, that man and his allergies are not unique.

The experiments reviewed here have been selected with a view to pre-

senting some of the means of experimental sensitization which may also operate in human allergies. The varieties of the manifestations of experimental anaphylaxis have been described with the purpose of bringing out similarities between these diseases and the natural allergic diseases of man.

No attempt has been made to discuss the most important question of the relation of the reagins or passively sensitizing antibodies found in allergic diseases to those antibodies which operate in the passive sensitization of animals. This is a very crucial field of investigation, which is only just beginning to unfold and which would require a separate paper to present.

A few of the experiments discussed in the text are summarized for the purpose of re-emphasizing their interest to the allergist.

(1) Minute amounts of antigen, even less than  $1\text{ }\mu\text{g}$ , may suffice to sensitize an animal, and only slightly larger amounts can produce shock. Such small quantities of antigen are well within the range to which man is naturally exposed.

(2) Sensitization of a local area of the body can be established if antigen is concentrated in this area. One condition under which such concentration can occur is found if antigen is circulating at a time when a local focus of inflammation is present in the body. Circulating antigen may be concentrated in this local area.

(3) The amount of antibody necessary to establish hypersensitivity may be so small that *in vitro* methods cannot demonstrate its presence in the circulation. The *in vivo* tests for hypersensitivity are much more sensitive than the *in vitro* tests.

(4) Both sensitization and shock can be experimentally elicited by ingestion or inhalation of antigen as well as by parenteral administration.

(5) The local and systemic effects of hypersensitivity may be profoundly damaging and simulate such chronic diseases in man as periarteritis nodosa, nephritis, and multiple sclerosis, as well as heart lesions not unlike those of rheumatic fever.

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# A CLASSIFICATION OF ALLERGIC DISEASES AND THEIR SPECIFIC MANIFESTATIONS IN ANIMALS

By Lester Reddin, Jr.

*Merck and Company, Westfield, New Jersey\**

## *Hypersensitivity*

In order that we may consider adequately the identity of certain manifestations of the allergic reaction as appearing in animals, it would be well, first of all, to develop a suitable working classification of this group of disturbances and its relationship to other physiologic phenomena.

So much has already been entered in the annals of modern medicine on the historical aspects of the field that only the salient points necessary to establish our logic in placing each of the subdivisions in its proper category need be mentioned here. Among the controversial issues in our medical concepts of today is that of the relationship of one manifestation of specific sensitivity to another. As in other fields where the thinking is healthy and progressive, these issues are vigorously expressed and as firmly held.

Our early knowledge of sensitivity did not allow us to speculate as to the mechanism of the reaction. It was in 1902 that Richet and Portier<sup>47b</sup> gave the name "anaphylaxis" to a peculiar finding in their experimental dogs. They observed that dogs which had survived previous injections of extracts of certain actinians would suffer on subsequent injections, with a syndrome of characteristic symptoms, and would rapidly succumb. They had expected that the previous injection would produce a state of resistance rather than an increase in susceptibility. It was this lack of resistance that caused them to coin the term "anaphylaxis": without protection.

The modern concept of this altered state can be based on the report of von Pirquet and Schick<sup>40</sup> on serum sickness. Although this report did not, at the time, prove the antigen-antibody interreaction as the etiologic factor, these workers are to be credited with postulating its existence before the mechanism of anaphylaxis of animals had been worked out. The dependence of anaphylaxis on antibody was demonstrated by Otto<sup>39</sup> and by Friedemann<sup>21</sup> in the guinea pig, by Richet<sup>47a</sup> in the dog, and by Nicolle<sup>28</sup> in the rabbit by injecting the serum from a sensitized animal into a normal one and showing the latter, on the following day, to be hypersensitive to the homologous antigen.

The existence of the hypersensitive state in the human being had long been known or suspected, but it was not until 1921 that evidence was presented to establish the antigen-antibody mediation of the phenomenon. It was in this year that Prausnitz and Küstner<sup>41</sup> published the results of their brilliant observations of the passive transfer of sensitivity to non-sensitive subjects by way of the serum.

Thus, the establishment of the antigen-antibody relationship of these conditions, *i.e.*, anaphylaxis of animals and allergic diseases of man, prompted Coca<sup>12</sup> to consider these two diseases in relation to the field of

\* Present address: Lawrence Veterinary Hospital, Verona, New Jersey.

immunology as a whole. His classification, with some extension, may be illustrated by TABLE 1.

It is from a casual standpoint only that we are interested at the moment with the top of this table, and then only in order to orient our thinking. It is, on the other hand, with the subdivision of the group of immunological diseases, hypersensitivity, that we will deal (See TABLE 2).

Hypersensitivity has been defined by Coca<sup>10</sup> as follows: "If an individual reacts specifically with characteristic symptoms to the administration of, or to contact with, a quantity of any substance, which, to the majority of

TABLE 1  
Immunology

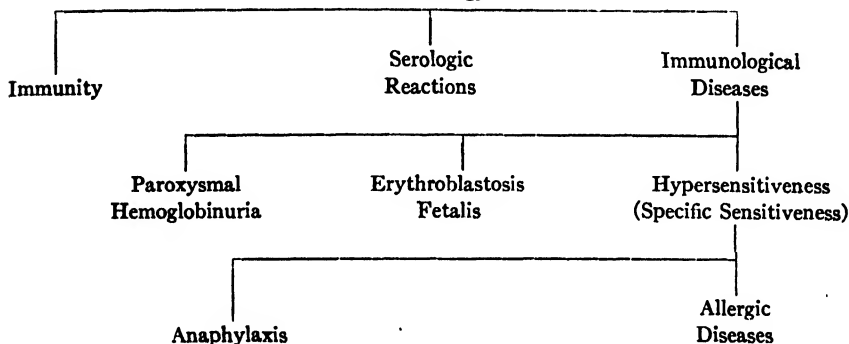


TABLE 2

<i>Specific Sensitiveness</i>	
A. Anaphylaxis	B. Allergic diseases
	1. Atopy-reaginic allergy
	2. Contact dermatitis
	3. Serum sickness
	4. Drug allergy
	5. Hypersensitivity of infection
	6. Non-atopic or non-reaginic allergy

the members of the same species of animal that have not had previous contact with it is innocuous, that individual is said to be hypersensitive to that substance."

It is unfortunate, from an etymological point of view, that the term "hypersensitive" should have received such wide usage, since it denotes an increased state of sensitivity, thereby implying that all individuals are sensitive to some degree. It would, on the other hand, seem better to adopt the term "specific sensitiveness" as suggested by Coca.<sup>10</sup>

### *Anaphylaxis*

For years, in many circles, the words "anaphylaxis" and "allergy" have been used synonymously. This interchangeable usage, however, has not

been borne out by experimental and clinical evidence. Anaphylaxis can best be described as a state of specific sensitivity of animals, induced either by previous contact with the antigen or passively with specific antibodies for it from another individual and mediated by the presence, in certain tissues, of anaphylactic antibodies. These antibodies are precipitins. They have the capacity of sensitizing smooth muscle and can be classically demonstrated by the Dale method of contraction of the isolated uterine strip.

That these antibodies are not the same as those found in atopy and other manifestations of allergic diseases of man and animals will be shown in the discussion under these other headings.

### *Allergic Diseases*

*Atopy.* In its earliest definition, atopy encompassed "certain clinical forms of human hypersensitiveness that do not occur, so far as is known, in the lower animal and which are subject to an hereditary influence."<sup>16</sup> Later developments have changed our views somewhat to establish its existence in lower animals and its mediation by the sensitizing antibody reagin.

Subsequent to the suggestion of Wolff-Eisner,<sup>58</sup> and later Meltzer,<sup>37</sup> that hay fever was an expression of hypersensitiveness, many unsuccessful attempts were made to transfer an antibody to the guinea pig by way of the serum of the sensitive individual. These efforts were directed toward the duplication in the guinea pig of the classical anaphylactic syndrome.

As mentioned earlier, an antigen-antibody concept of the mechanism of hay fever was suspected. This theory was given further credibility by the development of the technique of skin testing for hypersensitivity to inhalants. It was not until 1921, however, that the presence of antibody of the specifically sensitive individual was shown. In that year, Prausnitz and Küstner<sup>41</sup> succeeded in passively inducing a state of local sensitivity in the normal human skin following the injection of serum from a sensitive subject. Specific antibodies were later characterized in atopic sera and given the name "atopic reagin" by Grove and Coca.<sup>25</sup> Conditions in the human subject characterized by the presence of these sensitizing antibodies (*i.e.*, reagins) are, for example, certain forms of asthma, hay fever, and eczema. The identity of the mechanism of anaphylaxis of animals and asthma of humans was theorized by many, but consistent experimental proof failed to be developed to warrant any such similarity.

In order to discuss further the difference in the two phenomena, reference can well be made here to a comparison of the two types of antibodies to a single antigen (TABLE 3). Although these various points of differentiation have been open to criticism by many workers, the bulk of information recorded will be seen to bear out their accuracy. Because of the many published examples of this positive proof, only a few will be cited here.

Studying the sensitizing properties of a serum from an atopic subject, Donnally<sup>19</sup> showed that it was capable of sensitizing human skin in quantities as small as  $\frac{1}{100}$  cc., whereas Coca and Grove<sup>15</sup> have cited the consistent failure of the anaphylactic antibodies to sensitize human skin in any

concentration. The permanent attachment of the atopic reagins to the body cells at the site of injection has been demonstrated by Coca and Grove,<sup>16</sup> who have shown that this attachment can be observed for at least four weeks. This is in contrast to the rapid diffusibility of anaphylactic antibodies from their site of injection. It has been shown that this will occur in the rabbit within eighteen hours after injection. Using the technique of Dale, it has been consistently shown that anaphylactic antibodies are capable of passively sensitizing guinea pig uterine strips, while Prausnitz and Küstner,<sup>41</sup> Cooke, Flood, and Coca,<sup>17</sup> and Coca and Grove<sup>16</sup> were never able to accomplish this, using human atopic serum.

DeBesche<sup>2</sup> has shown that, although precipitins may on occasion be found to be present in the sera of atopic individuals, these antibodies fluctuate above and below the level at which they are demonstrable *in vitro*. Cooke, Flood, and Coca have further pointed out that the presence of these antibodies in human serum can in no way be demonstrated by passive transfer to the guinea pig, even by the very delicate technique of Dale, while, at the

TABLE 3

<i>Atopic reagins</i>	<i>Anaphylactic antibodies</i>
1. Sensitize human skin in as little as 1/200 cc.	1. Do not sensitize human skin in any quantity.
2. Are quickly and permanently attached to body cells.	2. Are diffused throughout body from injection.
3. Do not sensitize guinea pigs.	3. Sensitize guinea pigs.
4. Are not precipitating antibodies.	4. Are precipitating antibodies.
5. Typically do not inactivate antigen.	5. Inactivate the antigen.
6. Are susceptible to heat (destroyed at 56°C. for 1/2 hour).	6. Only slightly affected by 1/2 hour at 56°C. heat.

same time, Grove<sup>24</sup> has demonstrated that rabbits whose serum showed satisfactory precipitin titers would invariably die in anaphylactic shock when egg white was injected intravenously immediately after the blood titrations had been made.

Another criterion in the differentiation of these two antibodies was established by Levine and Coca<sup>35</sup> and confirmed by Jadassohn.<sup>28</sup> These investigators have shown that, while the atopic excitant is capable of completely inhibiting the sensitizing power of the reagin, the reagin lacks the property of inactivation of the atopen. In a recent study of the immunologic properties of the bovine reagin of ragweed sensitivity, the writer<sup>45</sup> has found a similar condition to exist in this species. In contrast to this, it is well known that anaphylactic antibody is completely neutralized by its corresponding antigen. The destruction of the atopic reagin by heat for one-half hour at 56° C. and the only slight alteration in the anaphylactic antibody by the same treatment was described by Coca and Grove<sup>16</sup> and later confirmed by Jadassohn.<sup>28</sup>

*Contact Dermatitis.* Allergic contact dermatitis is the phenomenon of altered reactivity of the skin caused by first contact with a sensitizing substance and manifested after an interval upon further surface contact with the original substance. In this category are included those sensitivities

formerly classified as dermatitis venenata, which is an eruption due to contact with poison ivy, poison oak, poison sumac, and other plants, and contact sensitivity to chemical substances. The absence of effect after first contact indicates that the plant oils or chemicals are not primary irritants. The appearance of dermatitis after subsequent exposure, however, establishes the allergic character of the condition.

In order properly to distinguish this group from various eczemas due to the atopic influence, a few points of differentiation can be made. That the atopic condition is influenced by heredity was early shown by Bonne,<sup>4</sup> Galewsky,<sup>22</sup> Darier,<sup>18</sup> and Brocq.<sup>6</sup> In fact, Jordan<sup>20</sup> was able to trace the transmission of atopic eczema through four generations. In contrast in contact dermatitis, stands the work of Straus,<sup>52</sup> who was able to sensitize the skin only after two or three surface applications of an extract of poison ivy. This lack of hereditary influence is further borne out by the work of Brown, Milford, and Coca,<sup>7</sup> who found that the incidence of success in sensitizing non-atopic skins is the same as that achieved in sensitizing atopic ones.

The presence of demonstrable reagents in atopic eczema and their absence in contact dermatitis have been reported repeatedly when using the transfer technique of Prausnitz and Kùstner (Coca and Grove<sup>14</sup> and Bloch<sup>3</sup>). However, the presence of some sort of antibody has been suggested to be instrumental in contact dermatitis by the work of Urbach,<sup>64</sup> who has reported that vesicatory fluid from eruptions due to contact allergy is capable of inducing a passive sensitivity in normal skin.

It has often been shown that there is a necessity for direct surface application of the allergen in contact dermatitis, since positive reactions are never obtained through the use of intracutaneous injections. This, of course, is in direct contrast to the sensitivity of the atopic skin to the scratch, intracutaneous, and percutaneous (Herrmann<sup>27</sup>) methods of testing.

*Serum Allergy (Serum Disease).* The term "serum sickness" was presented in 1905 by von Pirquet and Schick<sup>40</sup> to describe the symptom complex developing after a primary injection of a foreign serum. It was early noted that these undesirable reactions, including urticaria, fever, rash, adenopathy, and others, were not evident immediately following the primary injection but, rather, were displayed some 8 to 12 days thereafter. This delay or incubation period has served as one of the differentiating features of the disease. Prior to the report of von Pirquet and Schick,<sup>40</sup> no generally acceptable explanation for the mechanism of the condition had been presented. In their monograph, however, they proposed that the reaction was due to the effects of a toxic substance that resulted from the interaction of a newly formed antibody with residual amounts of the antigen, *i.e.*, foreign serum, still present in the circulation. In the experimental evidence brought forth by these workers, it was further shown that, following a second injection into rabbits, the incubation period was either lacking or greatly shortened. They therefore suggested that this reduced incubation period was due to the presence of a preformed antibody from the previous injection. In their efforts to explain the observations on the basis of an antigen-anti-

body theory, these workers did not credit the precipitin as the mediating antibody. Indeed, they explicitly postulated some other unidentified antibody in this connection, pointing out that precipitin was not consistently present. This fact was further emphasized by the report of Tuft and Ramsdell<sup>58</sup> in 1929, which showed the almost complete absence of precipitins and anaphylactic antibodies in individuals who had received large doses of normal horse serum, even though many of them developed serum sickness. This was confirmed by Coca, Deibert, and Menger<sup>14</sup> by examining at two-day intervals the serum of 26 subjects to whom had been administered sizeable amounts of normal horse serum. In no instance were precipitins found, in spite of the fact that some of the subjects developed serum disease.

Fleisher,<sup>29</sup> studying serum sickness as occurring in the rabbit, concluded that "it has not been possible to demonstrate in rabbits affected with serum sickness any constant temporal relationship between precipitins and precipitinogens in the blood on one hand and of the occurrence of serum sickness on the other hand." That the mediating mechanism of the condition is, however, an antibody of some type, even though not precipitin, has now been established by Karelitz and Stempien<sup>31</sup> by using the technique of Voss. They have shown that serum from patients recovering from serum sickness exhibits the power of inducing an immediate attack of the disease when administered to horse-serum-treated individuals.

*Drug Allergy.* Closely allied to the foregoing serum disease is the condition of specific sensitiveness to drugs. This condition is exhibited in the sensitive individual by an unusual yet characteristic response to a drug, which response is lacking in most individuals. That these two reactions are closely related is evident from the facts that a common incubation period must transpire before symptoms are displayed and the almost identical list of symptoms is present in both diseases. The one stumbling block at the present in ascribing the same mechanism to both phenomena is the lack of antigenic properties of most of the excitants of the drug allergies.

*Hypersensitiveness of Infection.* That altered response to bacterial infections may be manifested in several forms within the body has been pointed out by several writers. Scherago<sup>48</sup> has cited bacterial hypersensitivity of the atopic type exhibiting the immediate type of reaction following intradermal exposure to filtrates or products of bacteria, bacterial anaphylaxis, and the tuberculin type of sensitivity. The mechanism of the first two is, of course, the same as that responsible for those types of sensitivity to other antigens.

The tuberculin type of specific sensitivity, on the other hand, is different from these two by the following distinguishing features:

- (1) When the products, *i.e.*, filtrates, sonic extracts, *etc.*, are injected into the skin, instead of an immediate erythematous wheal and hyperemic flare being developed, as in simple atopy, a delayed inflammatory reaction sets in after several hours and progresses to reach a maximum size and intensity in 24 to 48 hours and then slowly fades by 72 or 96 hours.

- (2) The reaction of the tuberculin type sensitivity is more severe, being indurated with some erythema as against the soft edematous reaction of atopy.
- (3) The Prausnitz-Küstner technique of passive transfer of sensitivity cannot be demonstrated with the serum of tuberculin-sensitive subjects.
- (4) Body cells from the tuberculin-sensitive individual are killed *in vitro* by exposure to the bacterial protein, whereas cells from the atopic- or anaphylactic-sensitive individual are not killed by contact with the specific agent *in vitro*.
- (5) When the antigen is injected either subcutaneously or intravascularly in the tuberculin-sensitive subject, a slow reaction of severe illness and occasional death may take place. Illness does not start until some hours after the exposure to the antigen and death does not occur before eighteen to twenty-four hours. This is in contrast to the immediate collapse and death observed in anaphylactically sensitive individuals.
- (6) Whereas, ordinarily, contact with any foreign soluble protein will induce anaphylaxis in the guinea pig, to produce the tuberculin type of sensitivity it is necessary for the animal to have had previous contact with the intact organism or virus before sensitivity to an extract can be demonstrated.
- (7) There is a wide distribution of body cells which are tuberculin-sensitive, while unstriated muscle is the only shock organ in anaphylaxis.

*Familial Nonreaginic Allergy.* The oft-encountered negative reactions to diagnostic tests in known food allergic individuals long remained unexplained. A lead to the understanding and the development of a suitable diagnostic criterion came unexpectedly through a chance observation.

While studying the characteristics of a case of angina in which attacks had been precipitated by therapeutic doses of dilaudid, Coca<sup>11</sup> observed such a rapid heartbeat that the pulse could not be counted. Soon afterward, he noted in this individual that the symptoms of angina, as well as the accelerated pulse, followed the ingestion of certain foods. This observation was made in 1935. Since that time, many similar cases of suspected food allergies with the absence of positive skin tests have been examined. It has been noted that this specific tachycardia is consistently present. Coca was further able to show that the atopic reagins were absent by virtue of negative skin tests, as well as failures to transfer passively any sensitivity by the P K technique. This consistent absence of reagins prompted Coca to designate the class of disturbances as nonreaginic food allergies. This condition has been shown to be free of the atopic influence, even though the atopic shock organ may at times be affected. Examples of such occurrences are the so-called intrinsic asthmas and the group of nonreaginic eczemas.

#### *Allergic Diseases of Animals*

With the classification as a background, it is appropriate now to consider the diseases of lower animals in relation to the phenomenon of hypersensi-

tivity, so let us discuss the few conditions of allergic manifestations of domestic animals that have been described.

*Atopy.* Early work indicated that lower animals were capable of becoming experimentally sensitized and of showing signs of asthma after exposure to an offending allergen.

In an extensive study, Ratner<sup>44</sup> was able to demonstrate sensitivity in guinea pigs induced entirely by inhalation of dust antigens. The animals were exposed to the materials for varying lengths of time and observed for clinical evidence of sensitivity. In many of the guinea pigs thus observed, a syndrome of respiratory difficulties was induced which very strongly resembled clinical asthma of man. It is interesting to note that in some of the guinea pigs which did not develop this pulmonary involvement, anaphylactic sensitivity was, however, produced. Conversely, not all animals displaying the asthmatic seizures were capable of being thrown into anaphylactic shock. This paper is of especial interest, for it laid the basis for an understanding of clinical sensitivity of animals as induced by inhalation.

The first indications of atopic sensitivity in our domestic animals as indicated by positive skin reactions were presented by the reports of Schnelle,<sup>40</sup> Burns,<sup>9</sup> and Pomeroy.<sup>42</sup> These workers individually attempted to correlate the results of positive skin tests to certain food extracts with the presence of clinical evidence of sensitivity. Schnelle, in 1933, described the results obtained from applying intracutaneous tests with salmon and corn meal in two dogs known to be subject to eczema. Both of the animals reacted to the test injections, as well as to potato and wheat flour in one and pork and rice in the other. These positive reactions were further shown to be significant by trial feedings with diets containing these allergens. Typical eczema was produced in the subjects in three and six days following the start of the feeding tests. Although Burns did not use standardized extracts of a specific protein content, he was able to show that there were definite signs of allergic sensitivity to the foods after ingestion. Among the offending agents which he found to cause positive skin reactions upon injection in his patients, rice and tomatoes were found, upon feeding trials, to cause symptoms of gastro-intestinal disturbance as well as dermatitis of varying severity.

Little appeared in the literature for several years thereafter on atopic conditions in animal disease until 1941, when the report of Wittich<sup>57</sup> was received with interest. It was in this report that he described the first recorded analysis of the allergic mechanism of asthma in the dog. Wittich was able to establish the seasonal appearance of the symptoms, positive skin reactions to the inhalant, and passive transfer of the sensitizing antibodies to the skin of a non-sensitive animal of another breed. The sensitive animal had been suffering from seasonal attacks of sneezing, tearing, conjunctival injection, nasal blocking with watery discharge at times, and small circumscribed raised swellings about the face and body. These swellings were the cause of much violent scratching, resulting in large excoriations of the skin. Because of the owner's habits, the animal was usually removed in the summer to an area near a lake shore where the



incidence of pollenosis was low. At these times, there was a remission of symptoms in the dog. However, when she was brought back to her city home, the return was accompanied by an exacerbation of symptoms. Skin tests with the pollens most frequently involved in the patient's area resulted in positive reactions to giant and short ragweed, pigweed, prairie sage, and Russian thistle. The short ragweed and prairie sage produced the largest wheals with pseudopod formation. Shortly following the appearance of the positive skin reactions, signs of general disturbances were noted, including those symptoms formerly mentioned. These were promptly relieved by the administration of epinephrine by injection and inhalation. The further establishment of the atopic character of the attack was achieved by successful passive transfer of the sensitivity to a non-sensitive dog and human subject. During the following years of the animal's life, she received desensitizing injections of mixed pollens, to which she had shown positive reactions. Following the inception of this method of treatment, and until the death of the dog, no further symptoms of the sensitivity were observed.

No further evidence of atopic sensitivity in domestic animals was presented until 1943, when Weil and Reddin<sup>46</sup> reported on the existence of dermal supersensitivity to ragweed in a herd of cattle under their observation.

These workers established the presence of a sensitizing reagin which was capable of inducing sensitivity in the skin of a non-sensitive cow upon intradermal injection. This antibody was shown to be similar to the human ragweed reagin in that it was destroyed by exposure to heat at 56°C. for 2 hours. This study further revealed that the bovine species was able to produce a neutralizing antibody upon immunization with the specific antigen. It is not destroyed by exposure to a temperature of 56°C. and also can be produced by successive injections of the antigen in the non-sensitive subject.

In a later paper, Reddin<sup>46</sup> showed further that the properties of the reagin were identical with that found in human ragweed sensitivity, at least as far as certain immunologic properties were concerned. This study showed that the reagin was specifically inactivated by the corresponding antigen, even though it was unable to neutralize the antigen. It was further shown that reagin cannot be produced by continued injection of a non-sensitive cow with the specific antigen.

While data was being gathered for the second report, the entire herd was examined for the existence of dermal sensitivity, and it was found that 40 per cent of the animals exhibited some degree of reaction. The three cows which gave the strongest skin reactions were tested for ophthalmic sensitivity by insufflation of dry ragweed pollen into the conjunctival sac. Only one of these showed lacrimation and mild injection of the conjunctival vessels. During the following ragweed-pollen season, however, no signs of clinical sensitivity were observed.

The question of the existence of clinical pollenosis in cattle still remains unanswered. However, the ophthalmic reaction here cited would seem

to make the answer more than theoretical. In this connection, one should recall the cases of reputed typical hay fever in pure-bred cattle, as cited by Bray<sup>6</sup> in England. His report is, however, lacking in serological analysis, the diagnosis having been made on seasonal occurrence of symptoms only.

In the interim between these two reports on the bovine reagin, another example of reaginic sensitivity in the lower animal was presented. In 1944, Brunner and co-workers<sup>8</sup> were able to demonstrate the natural presence of skin-sensitizing antibodies in dogs, as evidenced by positive skin reactions to an extract of an ascarid. They further showed that, in the animals which were harboring these nematodes in their intestinal canal, the serum contained the antibody, and that it was capable of passively sensitizing the skin both of non-sensitive canines and of human subjects.

*Contact Dermatitis.* Landsteiner and Chase<sup>33, 34</sup> have been able experimentally to demonstrate contact dermatitis due to simple chemical substances and poison ivy in the guinea pig. Simon and others<sup>51</sup> have also experimentally produced dermatitis caused by poison ivy in the guinea pig, as has Straus<sup>52</sup> in the monkey. To my knowledge, however, naturally occurring contact dermatitis in lower animals was not reported until 1946. In that year, Reddin and Stever<sup>46</sup> presented a case report of a horse with an extensive dermatitis of three years' duration. The animal affected was a fine hunter-type horse which had received the best possible care. The skin had been treated by several veterinarians in various parts of the country without satisfactory results. The lesions were small raised areas, well circumscribed and apparently limited to the epidermis. They occurred over the neck, shoulder, and costal regions.

The location of these lesions suggested that there was a possible association with some substance used either in the tanning of the leather of the saddlery or in its cleansing or conditioning. It had been the practice, in this particular stable, to wash the saddlery with a popular saddle soap, followed by the application of a well-known leather conditioner. Inunction tests with these products separately caused no reaction to normal areas of the skin, but, when mixed and rubbed into the skin, pronounced local swelling resulted some hours thereafter. When the individual ingredients of the two products were tested both singly and in combination, negative reactions resulted, except in the case of a combination of one oil and a dye. The pigment, "wool yellow dye," and sulfonated neatsfoot oil, when mixed and applied, proved to be the offending substances. Relief was afforded by application of a bland oil preparation to the affected areas. Subsequent attacks of the dermatitis were avoided following the thorough cleansing of the leather and the use of a saddle soap which did not contain the pigment.

*Serum Sickness and Drug Allergies.* The earliest report of serum sickness occurring in animals is that of Bécère, Chambon, and Ménard<sup>1</sup> published in 1896. These writers noted the appearance of varied types of eruptions, fever, and evidence of disturbance of locomotion in cattle about four days after the injection of large amounts of horse serum.

Gerlach<sup>35</sup> reported, in 1922, the occurrence of symptoms of serum sickness

in horses and cattle upon injection of a heterologous serum. He found that the reactions in horses were less severe than in cattle and that the size of injection had little influence upon the severity of the attacks. A further observation made by this worker was that the sensitivity could not be transferred to the guinea pig by way of the serum from an affected animal, thus showing that he was dealing with serum sickness rather than with anaphylaxis.

In an extensive series of reports, Fleisher and his co-workers<sup>20, 26, 29</sup> have described the manifestations of experimentally-induced serum sickness in the rabbit. They have described the disease in two forms, that is, the delayed and accelerated types. The delayed reaction followed a primary injection of the foreign serum in from 3 to 8 days, while the accelerated reaction set in within 8 to 72 hours after a second injection. The symptoms most frequently observed in their rabbits included erythema and edema of the ears. The erythema was of the morbilliform or scarlatinal type. It was shown in these studies that there was no constant relationship between the presence of precipitins in the serum of the affected rabbits and the development of serum sickness. It was further found that there was an elevation of body temperature in about 53 per cent of the rabbits which developed the disease, but in those injected with the foreign serum that did not develop serum sickness only 22 per cent showed any degree of hyperpyrexia. Changes in the white cell content of the blood of afflicted rabbits were not constant, although there was occasionally a mild leucopenia associated with the appearance of the disease.

The incidence of allergies to drugs in domestic animals has received little attention. However, the increased use of antibiotic and chemotherapeutic agents in veterinary medicine can be expected to shed some light on the problem. That some allergic manifestation may at times appear to be the result of drug administration has been suggested by the reports of Klein<sup>32</sup> and Stubbs<sup>36</sup> and their co-workers. In the study of the pharmacology of sulfanilamide, Klein observed an urticaria in one cow. Subsequently, Stubbs used the same animal in studies involving sulfathiazole, and again observed a reaction of similar nature following the use of this second sulfonamide.

*Non-Reaginic Food Allergy of Animals.* The diagnosis of non-reaginic allergy, based primarily upon the change in pulse rate within a short time after exposure to the allergen, presents a particularly difficult problem to the veterinarian in clinical practice. Lower animals are subject to emotional disturbances which readily affect the heart rate, thereby confusing the interpretation of the test. Even though the use of this classical diagnostic test will be highly restricted, some progress may be expected by careful observation when employing elimination diets.

Some case reports from the literature are of interest in this connection. A baby walrus was captured in the Bering Sea and was bottle fed on cows' milk. Small eroding areas, which sometimes bled, appeared on her head and flippers. She developed a mild conjunctivitis and there was increased salivation accompanied by drooling. The feces were abnormally soft and flecked with mucus.

Schroeder<sup>50</sup> systematically studied the case. Changes in the diet were made to determine whether the condition represented nutritional deficiency. Careful observation of the lesions established that they were more severe shortly after each feeding. This led him to consider the possibility of an allergic reaction being involved. Withdrawal of the cows' milk was followed by an almost immediate remission of the severe signs. The lesions healed and the skin became smooth and dry, although scars remained.

Very recently, Povar<sup>48</sup> has presented a report of a series of cases which exhibited various signs of food allergy in dogs. Some dogs were affected with a hemorrhagic colitis of varying intensity. The condition appeared and disappeared rapidly, except in a few cases, where death resulted within 24 hours after the onset of hemorrhage. Death was prevented in other severe cases by resort to transfusions. Pathologic examination of one of the fatal cases revealed extensive submucous hemorrhage with cellular filtration of the colon. The blood vessels of the entire thickness of the intestinal wall were congested and dilated, while some vessels contained "an organized mass of blood clot which was granular and necrotic in appearance and extensively infiltrated with polymorphonuclear leukocytes." In some of the cases which did not terminate fatally, avoidance of horse meat and, in others, commercially prepared food resulted in the disappearance of symptoms.

The nature of the symptoms cited by these two writers and their similarity with symptoms observed in some cases of non-reaginic food allergy of man has led Coca<sup>13</sup> to believe that allergies of this classification do occur in lower animals.

The recognition of allergic diseases in lower animals has not been frequent. Since one or two examples of each class of allergy have been reported, however, the fact is established that lower animals possess the capacity to develop allergies with the same characteristics as those observed in man. Careful observation and the use, so far as is possible, of diagnostic tests may be expected to add additional examples. The importance of the field is self-evident, as recently expressed by Weil<sup>55</sup>: "Altogether a whole nearly unexplored field of research is open here for the mutual benefit of human and veterinary medicine, promising a harvest valuable in itself and for the sake of creating tools for experimentation."

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## SERUM SICKNESS .

By Samuel Karelitz

*The Willard Parker Hospital and the Mount Sinai Hospital, New York*

The term "serum sickness" was coined by von Pirquet and Schick<sup>1</sup> to describe the reaction of man to the injection of a heterologous animal serum. For 8-12 days after the injection of animal serum (horse) nothing abnormal is noted. After that, the patient with manifest serum sickness develops a rash, most often urticarial; fever; lymph-gland enlargement; edema; arthralgia; and leucopenia with a relative lymphocytosis. Von Pirquet and Schick described these signs and symptoms in great detail and ascribed them to a toxic substance which they believed resulted from a reaction between the antigen (horse serum) and antibody. This occurred only after the antibody had reached an adequate concentration. They noted that a second injection of the horse serum in the same person called forth another attack of serum sickness, but after a shorter period of incubation, 5-7 days (accelerated reaction), and in some cases an immediate reaction occurred within a few minutes. Von Pirquet and Schick demonstrated anti-horse serum precipitin in the blood of persons who were at the height of serum sickness, as did Hamburger and Moro.<sup>2</sup> Unlike the latter authors, von Pirquet and Schick did not think that precipitin was the antibody responsible for serum sickness, because some patients who developed serum sickness had no demonstrable circulating precipitin, while anti-horse serum precipitin was demonstrable in other patients who had been treated with horse serum but had not developed serum sickness. Furthermore, they were unable to demonstrate precipitin at the time of the immediate reaction which, in their opinion, was the most striking example of an antigen-antibody reaction.

Although the view that serum sickness is due to an antigen-antibody reaction seemed well supported by the clinical and experimental evidence presented by von Pirquet and Schick, this theory was questioned until Voss<sup>3</sup> demonstrated that serum sickness could be induced in horse serum-treated patients by injecting them with human serum obtained from persons convalescent from serum sickness caused by therapeutic horse serum. Harten and Walzer,<sup>4</sup> in their discussion of serum allergy, reviewed the work of Voss and of Szirmai<sup>5</sup> and all the available data pertaining to the antibodies obtained in serum sickness and concluded that the data failed to reveal a constant association of any type of antibody with serum sickness. They believed that the presence of antibodies in serum sickness convalescent serum (S.S.C.S.) had been assumed by Voss, since no antibody studies were reported. They agreed with the doubt previously expressed by Coca<sup>6</sup> that any circulatory antibody is responsible for serum sickness.

Since 1939, further studies<sup>7</sup> of induced passive serum sickness have been made. The results of some of these have already been reported. Coca<sup>8</sup> and Doerr<sup>9</sup> have accepted the findings as establishing the antigen-antibody nature of serum sickness.

The discussion which follows will present a brief summary of the observations reported by Voss and of our experience with passive serum sickness, reviewing both published data and those of unpublished studies carried out before 1942. An attempt will be made to establish the identity of delayed serum sickness and passively induced serum sickness and to show that passive serum sickness is caused by an antigen-antibody reaction, thus proving that delayed serum sickness is caused by an antigen-antibody reaction.

Finally, there will be a discussion of the antibodies detected in several specimens of S.S.C.S. and in sera obtained from horse serum-treated patients who had not yet developed serum sickness and the significance of these observations with regard to the nature of the serum sickness antibody.\*

*Observations of Voss.* Voss, encouraged by the successful use of measles convalescent serum in prevention of measles, attempted to prevent serum sickness in 36 children treated with diphtheria antitoxin 1-9 days before by injecting into them 2-10 ml. of serum obtained from patients convalescent from serum sickness resulting from diphtherial antitoxin (horse serum), S.S.C.S. He observed that the injection of convalescent serum 1-3 days following the intramuscular injection of diphtherial antitoxin resulted in the development of urticaria localized at the site of horse serum injection within 5-30 minutes. When the convalescent serum was given later in the incubationary period of serum sickness, constitutional reactions resembling anaphylaxis resulted. Delayed serum sickness occurred in only one of the 36 patients.

Voss gave the following explanation for this phenomenon. He assumed that the convalescent serum contained antibodies to horse serum, which, if supplied to a horse serum-treated individual, might raise the antihorse-antibody titre high enough to produce a serum reaction according to the theory postulated by Von Pirquet and Schick. The reaction would result in elimination of enough antigen to prevent the occurrence of the usual serum sickness. Furthermore, Voss believed that such a reaction was an example of human anaphylaxis, similar to the reversed anaphylaxis reaction of animals demonstrated by Opie and Furth,<sup>10</sup> Kellet,<sup>11</sup> and Zinsser and Enders.<sup>12</sup> Voss suggested that this procedure be employed to demonstrate humoral antibodies in man by a more localized reaction, which he developed. He produced such a localized reaction by the following technique: 0.1 ml. of 1/10 to 1/1000 saline dilution of horse serum is injected intradermally into a non-sensitive individual. After 8-24 hours 1-5 ml. of convalescent serum is injected intravenously. A large urticarial wheal, 1-5 cms. in diameter, develops in 5-30 minutes at the site of horse serum injection if specific humoral antibody for horse serum is present in the convalescent serum. This local reaction was reproduced by Szirmai and by Hopkins and Wright,<sup>13</sup> who found that it is suitable for the purpose of demonstrating antihorse-antibody in human sera.

\* Throughout this presentation, serum sickness will imply that it followed horse serum therapy. S.S.C.S., or convalescent serum, will signify human serum obtained during convalescence from serum sickness due to therapeutic horse serum. Passive serum sickness will indicate the serum sickness induced in horse serum-treated patients by the injection of S.S.C.S.

TABLE 1

EFFECT OF SERUM SICKNESS CONVALESCENT SERUM ON PATIENTS WHO WERE TREATED WITH IMMUNE HORSE SERUM

Number of cases	Type of serum therapy	Serum sickness convalescent serum		Passive serum sickness			Subsequent serum sickness
		Amount	Number	General	Local	None	
		ml.					
9	S.F.A. (B. of H.)	2-10	1	3	5	2	9
6	S.F.A. (B. of H.)	10-15	2	0	0	6	6
8	S.F.A. (B. of H.)	3-10	3	3	5	2	6
5	S.F.A. (B. of H.)	4-10	4	5	1	1	2
1	S.F.A. (B. of H.)	5	5	1	1	0	1
2	S.F.A. (B. of H.)	4.5	6	1	1	0	2
1	S.F.A. (B. of H.)	4	7	1	1	0	1
7	S.F.A. (B. of H.)	2-5	8	4	5	0	4
1	S.F.A. (B. of H.)	5	9	1	0	0	0
6	S.F.A. (B. of H.)	3-10	10	0	0	6	3
46	Total			19	18	17	34 or 73.9%
4	L.S.A.	2-10	1	0	2	2	0
1	L.S.A.	5	2	0	0	1	0
2	L.S.A.	5-10	3	0	0	2	0
1	L.S.A.	3	8	1	0	0	0
8	Total			1	2	5	0
2	C.S.F.S.	5-10	1	0	1	1	0
3	T.A.T.	2-5	4	1	3	0	0
2	Dip. A.T.	4-10	4	1	2	0	0
1	Influenza S.	2	5	0	0	1	1

Control Cases						
4	S.F.A. (B. of H.)	0.1-1 ml. H.S. in 10 ml. saline	0	0		2
3	S.F.A. (B. of H.)	10-20 A.S.	0	0		3
2	S.F.A. (B. of H.)	7 ml. C.S.F.S.				
		1 ml. S.F.A.	0	0		2
3	S.F.A. (B. of H.)	7-10 ml. C.S.F.S.	0	0		2
4	L.S.A.	10 ml. S.S.	0	0		0
2	Untreated scarlet fever	5 ml. S.S.C.S. #3	0	0		0
		5 ml. S.S.C.S. #4	0	0		0

S.F.A. (B. of H.), Scarlet Fever Antitoxin (New York Board of Health).

C.S.F.S., Scarlet Fever Convalescent Serum.

L.S.A., Refined Commercial (Lederle).

H.S., Horse Serum.

A.S., Human Adult Serum.

T.A.T., Tetanal Antitoxin.

D.A.T., Diphtherial Antitoxin.

S.S.C.S., Serum sickness convalescent serum.

*Review of Author's Data on Passively Induced Serum Sickness.* TABLE 17 reveals the statistical analysis of the results when, using 10 different convalescent sera, we attempted to produce passive serum sickness in 46 persons previously treated with scarlet-fever antitoxin, a horse serum prep-



aration consisting predominately of pseudoglobulin. The local reaction appeared in 18, and the general reaction in 19 cases treated with convalescent serum. Seventeen failed to show any reaction at all.

*Passively Induced Serum Sickness—General Reaction.* Clinically, the general reaction was typical in appearance to serum sickness. The rash was most commonly urticarial. The eruption first appeared at all sites injected with horse serum or at sites previously irritated by heat, cold, or xylol and was followed quickly by generalized itching, erythema, and urticaria. In two instances, a scarlatiniform rash appeared and in three, the urticaria was accompanied by angioneurotic edema of the lips and eyelids. In two cases, arthralgia was experienced and several developed fever. When the reaction lasted 24–48 hours, the passive serum sickness seemed to have initiated and merged with the delayed serum sickness, creating, in fact, an induced accelerated serum sickness. Both the urticarial and the scarlatiniform eruptions responded to adrenalin.

*Passively Induced Serum Sickness—Local Reaction.* The local variety of passive serum sickness conformed with that described by Voss. The localized urticarial wheal appeared 5 to 30 minutes after the injection of convalescent serum, except in an occasional case, when it appeared only after several hours. It occurred on all sites prepared with horse serum intradermally and lasted for about an hour.

*Specificity of Reaction—Horse Serum vs. Antihorse Antibody.* Specificity of the reaction for horse serum-treated individuals was shown as follows: (TABLE 1) S.S.C.S. induced the passive serum reaction when it was injected intravenously into patients treated 1–9 days before with tetanal antitoxin, diphtherial antitoxin, or scarlet-fever antitoxin, all horse serum preparations. However, this passive serum sickness was not induced in patients treated with human serum preparations, such as the scarlet-fever convalescent serum, and rarely in patients treated with refined scarlet-fever antitoxin prepared by pepsin digestion. Thus, it was shown that the antibody to horse and not an antibacterial antibody or antitoxin was significant in the production of passive serum sickness. Furthermore, it was not possible to induce passive serum sickness in patients treated with therapeutic horse serum (scarlet-fever antitoxin) 1–9 days before by the injection of normal human serum or normal horse serum. Serum sickness did not develop when cases of untreated scarlet fever received injections of serum sickness convalescent serum known to induce this reaction in patients previously treated with horse serum. Thus, it was also shown that passive serum sickness could be induced only in horse serum-treated patients when the S.S.C.S. which was used came from a patient who had had horse serum therapy.

*Incubation Period in Passive Serum Sickness.* Similarity between passive and delayed serum sickness was further advanced by showing that just as an incubation period is necessary for the production of delayed serum sickness, so was an incubation period found necessary for the production of passive serum sickness. This period was found to be in excess of one hour and less than 8 hours after treatment with horse serum.

Thirty-four children<sup>7b</sup> were treated with scarlet-fever antitoxin and with 5 c.c. of one of 4 specimens of serum-sickness convalescent serum simultaneously or within 5 minutes. Some of these children were injected intramuscularly with a combination of S.S.C.S. and the therapeutic horse serum mixed in a test tube immediately before the injection. Others were injected with therapeutic horse serum, and, through the same needle left *in situ*, the S.S.C.S. was promptly introduced. Others were simultaneously injected with the therapeutic serum in one buttock and the S.S.C.S. in the other. Passive serum sickness did not develop in any of these cases. In one child, local induration developed at the injected site, more suggestive of an inflammatory process than of an allergic response.

TABLE 2  
PASSIVE TRANSFER OF ANTIBODY TO HORSE SERUM IN S.S.C.S. BY TECHNIQUE OF VOSS

S.S.C.S.	Amt. injected	Positive reactions	Negative reactions
	ml.		
1	2-5	5	0
2	2-5	0	3
3	2-8	9	0
4	2-8	12	0
5	3-5	10	0
6	3-10	1	2
7	3-10	1	2
8	2-5	5	0
9	2-5	6	0
10	2-15	0	8
11	2-3	2	0
12	2-3	1	1
13	2-3	2	0
14	2	4	0
15	2	2	0
16	2	2	0
Total cases (78).....		62	16

*Passive Transfer Studies.* In subsequent studies,<sup>7c</sup> in order to further the evidence for the antigen-antibody theory of serum sickness, the passive transfer of the antihorse-serum antibody contained in S.S.C.S. was attempted. The techniques of Voss and of Prausnitz-Kuestner<sup>14</sup> in the usual and in the reverse order were employed. The technique of Voss is that previously described for demonstrating humoral antibodies in S.S.C.S.

TABLE 2 reveals the results obtained using the Voss technique with the first 16 sera studied. Using this technique, positive reactions were obtained in 62 out of 78 trials. Thus, transferable antibody to horse serum was demonstrated in 14 out of 16 sera. It is noteworthy that the sera which regularly failed to elicit this reaction are the same ones which previously failed to produce serum sickness. We have repeatedly reproduced this passive reaction more recently with other specimens of S.S.C.S. and with 3 sera obtained from horse serum-treated patients who did not have clinical serum sickness.

*Antigenicity of Normal Horse Serum and Fractionated Therapeutic Horse Serum for Passive Transfer Reaction.* Normal horse serum was found as effective an antigen as therapeutic horse serum for skin preparations in demonstration of this passive transfer antibody in S.S.C.S. If, instead of normal horse serum, highly refined diphtherial antitoxin was used to prepare the skin for the passive transfer, the reaction occurred less regularly and was weaker when it developed.

*Specificity of Passive Transfer Reaction.* Specificity of the reaction between horse serum and the S.S.C.S. was shown as follows: The skin of 2 children was prepared with 0.1 c.c. of hog, sheep, and horse serum, and the skin of 4 others with cat, dog, guinea pig, human, monkey, rabbit, and horse serum. Twenty-four hours later, 2-3 c.c. of S.S.C.S. was injected intravenously into each child. Typical reactions, large urticarial wheals, appeared only at the sites prepared with horse serum, except for one slight and delayed reaction which appeared at the site prepared with sheep serum and one at that prepared with dog serum.

*Passive Transfer by Technique of Prausnitz and Kuestner.* The sensitizing antibody contained in S.S.C.S. was again demonstrated by the technique of local passive transfer as done in the Prausnitz-Kuestner reaction. Local passive transfer of this antihorse-serum antibody was also shown by this technique performed in the reverse order, namely by preparing normal skin with 0.1 c.c. of horse serum and subsequently testing with 0.1-0.2 c.c. of S.S.C.S. Positive transfers were attained with 6 out of 7 S.S.C.S. tested. Each serum was tested on 3-9 individuals.

A larger reaction resulted at the transfer site if the human serum which was used as the antigen, instead of horse serum, was obtained from patients who had received therapeutic horse serum 48-72 hours previously. It was also observed that the reaction was more pronounced if the transfer was performed in the reverse order of the usual Prausnitz-Kuestner technique. This same observation has also been made by Wright and Hopkins.

*Effect of Heat on Passive Transfer Antibody.* Heating two of the S.S.C.S. #8 and #9 in a water bath for 90 minutes at 56°C. failed to interfere with the ability of these sera to give positive transfer tests. Likewise, heating another serum, #14, to 60°C. for one hour failed to destroy its ability to react at skin sites prepared with horse serum. These experiments demonstrated a transferable antibody to horse serum which was thermostable to 56°C. for 90 minutes and to 60°C. for one hour.<sup>15</sup>

*Skin Test for Horse Serum.* Since the horse serum injected into a person is quickly distributed to all parts of the body and can be demonstrated in the skin within 24 hours by the passive transfer techniques of Voss or by the Prausnitz-Kuestner reaction, the skin should contain sufficient antigen to react to an intradermal injection of S.S.C.S. containing antibody. It occurred to us, therefore, that it might be possible to demonstrate horse serum antibody contained in S.S.C.S. merely by injecting 0.1 or 0.2 ml. of this serum into the skin of individuals recently treated with horse serum. The results of the skin-test study are summarized in TABLE 3.

The skin reaction of untreated individuals to S.S.C.S. is similar to the re-

action to normal adult serum, *i.e.*, a raised blanched area about 0.5 cm. in diameter which rapidly disappears. The reaction to an intradermal injection of 0.2 c.c. S.S.C.S. by a patient previously treated with horse serum is a larger wheal, which becomes urticarial in a few minutes, forming pseudopodia, and is surrounded by erythema. The wheal grows to 1-5 cm. during the ensuing 5-30 minutes, then recedes to become completely absorbed in about one hour. This reaction can be elicited approximately one day following a horse serum injection and thereafter until the patient develops serum sickness, at which time the skin reaction is variable in intensity. If a patient is tested while having serum sickness, an urticarial wheal may form and blend with the urticaria of the serum sickness, the skin may swell and become erythematous, or there is little more than the usual reaction to normal horse serum. After the serum sickness has passed (also in patients who

TABLE 3  
SKIN REACTIONS TO S.S.C.S. #4 COMPARED TO HORSE SERUM IN PATIENTS TREATED WITH THERAPEUTIC HORSE SERUM

Days after horse serum therapy	Number of cases	S.S.C.S.		Horse serum	
		pos.	neg.	pos.	neg.
1	6	6	0	0	6
2	3	3	0	0	3
3-5	8	8	0	0	8
6-7	11	10	1	1	10
8	6	6	0	3	3
9	7	7	0	3	4
10	8	8	0	6	2
11	5	4	1	4	1
12	1	1	0	1	0
13	1	1	0	1	0
14	11	11	0	10	1
15	6	5	1	6	0
16-30	12	8	4	11	1

failed to develop serum sickness) the reaction to S.S.C.S. continues to be positive for a variable length of time after the injection of horse serum.

When the skin of a patient treated with horse serum is tested 24 hours later and daily thereafter with S.S.C.S. and simultaneously with horse serum, the reaction to the horse serum remains negative for about a week to ten days. During this same period of time the reaction to S.S.C.S. is positive (see FIGURE 1). The intensity of the reaction to S.S.C.S. increases for the first few days after horse serum therapy but becomes weaker when the horse serum begins to elicit positive reaction. After recovery from serum sickness and in horse serum-sensitive individuals, the reaction is usually more intensive to horse serum than to the S.S.C.S.

A skin area showing a positive skin reaction to horse serum ordinarily becomes positive again following an injection of horse serum into the same site on the next day. A site reacting to the S.S.C.S. shows little or no response to a second injection of S.S.C.S. injected into the same skin site the following day, while a skin site not previously tested gives a positive reaction.

The reactions to S.S.C.S. in an individual who has been previously treated with refined horse serum may be slight or negative. It was also noted that different batches of S.S.C.S. varied in their ability to elicit this reaction.

### Summary

Thus far, we have demonstrated that passive serum sickness is clinically similar to delayed serum sickness and that both are due to antigen-antibody

### CASES

A - Had Serum Sickness 11 - 12th days after therapy

B - No Serum Sickness

C - No Serum Sickness

D - Had Serum Sickness 10 - 12th days after therapy

Horse Serum - Thin line

S.S.C.S. - Heavy line

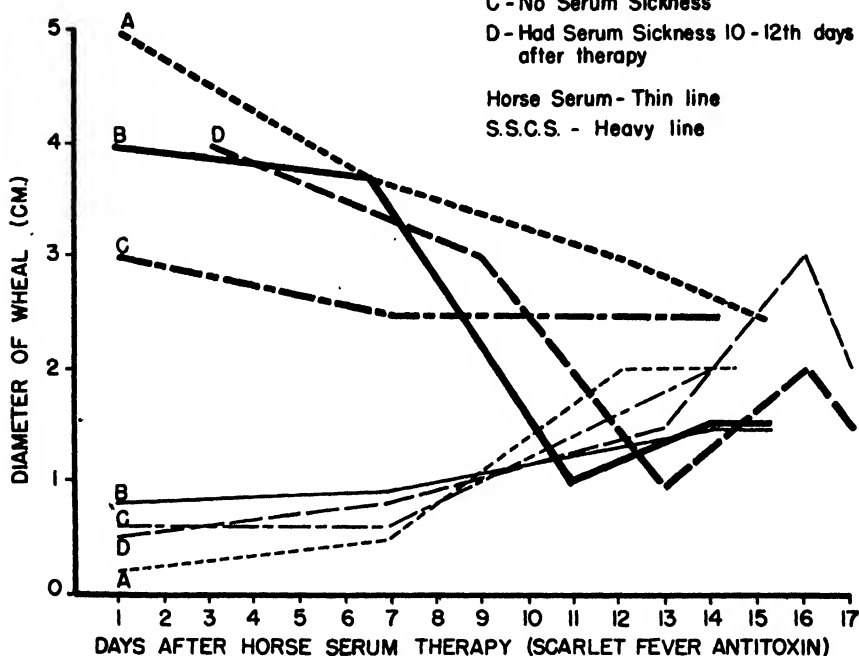


FIGURE 1. Skin test. Skin reaction of horse serum-treated individuals to intradermal injection of 0.2 ml. S.S.C.S. and comparison with skin reaction to horse serum.

reactions. An eruption, usually urticarial, fever, and joint pain are common to both, as is an incubation period. Passive serum sickness was shown to be a specific reaction of a horse serum-treated individual to convalescent serum obtained from patients who were treated with horse serum. Just as the incidence of delayed serum sickness is reduced by purification of the antiserum, so was the ability to reproduce passive serum sickness reduced by using purified therapeutic horse sera. It was possible to demonstrate this serum sickness antibody by the passive transfer reaction of Voss, by the local passive transfer reaction performed by the Prausnitz-Kuestner technique, and finally by the skin test.

*Analysis of Antibodies in S.S.C.S.*

The antigen-antibody nature of serum sickness having been demonstrated, an attempt was made to identify the antibodies contained in the S.S.C.S. and to determine the role played by them in serum sickness. Similar but less extensive analyses were made of several sera obtained from patients 14 days after they had received therapeutic horse serum, but who had not manifested clinical evidence of serum sickness. The results of these antibody determinations are presented in TABLES 4 and 5.

TABLE 4  
ANTIBODY ANALYSIS OF S.S.C.S.

No. of S.S. C.S.	Anti-horse pre- cipi- tative titer	Heterophile agglutinative titer			Produces passive serum sickness				Skin test in horse serum- treated cases	Pas- sive trans- fer (P.K.)	inverse passive trans- fer (P.K.)	Direct passive anaphylaxis in guinea pigs
					general		local					
		Non abs.	G.P. abs.	Beef RBC	pos.	neg.	pos.	neg.				
1	0	1/7	0	1/7	7	2	5	0	—	—	—	—
2	0	1/14	0	1/14	0	6	0	3	—	—	—	—
3	1/100	—	—	—	6	2	9	0	++	++	++	—
4	1/100	1/448	0	1/28	9	1	12	0	++++	++	++	Reaction & recovery
5	1/2000	1/14	0	0	1	1	10	0	—	—	—	—
6	1/50	1/28	0	1/14	2	0	1	2	++	+	+	Reaction & recovery
7	1/10+	1/14	0	1/14	1	0	1	2	+	+	+	Reaction & recovery
8	1/50± 1/1000	1/448	0	1/112	5	1	3	0	+++	++	++	Reaction & death
9	1/100	1/448	—	—	8	0	10	0	++	+	+	Reaction & death
10	1/1000	1/512	—	—	0	6	0	8	0	0	0	Reaction & death

Symbols: —, not done; +, positive; 0, negative; P.K., Prausnitz-Kuestner.

*Findings of Table 4.* Of the 10 different S.S.C.S. tested for precipitin by the ring test and with the collodion particle technique of Freund,<sup>16</sup> 2 gave negative reactions. Five out of 9 gave positive heterophile agglutinative reactions in titers of 1/28 or stronger, and 5 out of 7 of these sera tested for heterophile agglutinative antibody were slightly positive after absorption with beef erythrocytes. Passive serum sickness was produced with 8 of the 10 sera. Passive transfer of antihorse-serum antibody was accomplished with 6 of the 7 sera tested and the skin test was positive with the same 6 sera. Passive anaphylaxis in guinea pigs was demonstrated with all of the 6 sera tested.\*

All of the 6 sera which were shown to contain anaphylactin also contained

\* Techniques used: for each serum tested, 4 guinea pigs, each weighing about 250 grams, received subcutaneous injections of the S.S.C.S., and, after 18-24 hours, each received 2 cc. of horse serum intravenously.

precipitin, and only 5 of the 6 produced passive serum sickness. High precipitin titers did not always occur with high heterophile agglutinative titers nor with the ability of the particular serum to produce passive serum sickness. The sera with especially significant findings will be discussed individually.

Serum #1 gave a negative test for precipitin but produced passive serum sickness in 7 of 9 attempts and the local reaction of Voss in 5 out of 5.

Serum #5 caused irritation of the skin into which it was injected, making it difficult to interpret the passive transfer results.

*Antibody Reaction to Normal Horse Serum.* Serum #8 was obtained from a scarlet-fever patient who had recovered from serum sickness following an injection of 40 ml. of normal horse serum instead of therapeutic serum. Normal serum was given so that her antibody response could be followed on the basis of the observations made by Coca<sup>17</sup> and Tuft and Ramsdell<sup>18</sup> that the antibody response of healthy persons to normal horse serum was very weak even though these patients developed serum sickness. It is noteworthy that the serum sickness which this patient developed was accompanied by the development of antibodies in high concentration. The precipitin titer of her serum was positive against horse serum in 1/1000 dilution. The heterophile antibody titer was 1/448 non-absorbed and 1/112 after absorption with beef erythrocytes. Her serum produced passive serum sickness regularly.

The antihorse antibody contained in this serum could be transferred to normal skin. The serum produced the positive skin test in horse serum-treated persons and it caused passive anaphylaxis in guinea pigs. Possibly the difference between these observations and those made by Coca<sup>17</sup> after he had treated healthy American Indians with normal horse serum is that our patient was reacting simultaneously to a bacterial infection and to horse serum. Thus, possibly, an enhanced reaction to the horse serum resulted. Perhaps this might be compared to the induction of high precipitin and colloidal agglutination levels in guinea pigs by Freund and McDermott<sup>19</sup> by the injection of horse serum in a suspension of paraffin oil, falba, and killed tubercle bacilli.

*Antibody Response to Pepsin-Digested Scarlet-Fever Antitoxin.* Serum #10 was obtained from a patient whose serum sickness resulted from treatment with 15 c.c. of pepsin-digested refined scarlet-fever antitoxin. His serum sickness was moderate. The convalescent serum had a high titer for precipitin and heterophile antibody. It produced passive anaphylaxis in guinea pigs, yet, despite this and the high precipitin titer, it failed to induce passive serum sickness and passive transfer reaction or to produce a positive skin test in horse serum-treated individuals. The implications of these observations will be discussed later.

*Antibody Formation without Developing Serum Sickness.* The sera obtained from 3 patients, 14 days after they had been treated with scarlet-fever antitoxin, yielded significant findings (TABLE 5). These patients did not develop clinical serum sickness. The serum of 2 of the 3 had no demonstrable precipitin; yet all produced the local passive serum reaction. All had heterophile antibody but their nature was not determined.

*Antibody Response in Horse Serum-Sensitive Person.* Serum #14 was obtained from a 12 year-old colored boy who was ill with scarlet fever. Five minutes after 0.5 c.c. of a 1/10 dilution of scarlet-fever antitoxin was injected intradermally for a blanching test, he began to complain that his skin was hot and itching. Generalized urticaria with angioneurotic edema developed, especially at the eyelids, tongue, and pharynx. He became dyspnoeic, then unconscious. Artificial respiration and oxygen inhalations were given. Ten milliliters of calcium gluconate were injected intravenously and epinephrin intramuscularly and intravenously. He emerged from the stupor and two hours after his episode he felt well subjectively. However, his eyelids remained swollen for 24 hours. No further evidence of serum sickness developed. It was learned later that he was an asthmatic, but sensitivity

TABLE 5

ANTIBODIES DEMONSTRATED IN SERUM OBTAINED FROM PATIENTS (11, 12, 13) WHO WERE INJECTED WITH THERAPEUTIC HORSE SERUM 12-14 DAYS BEFORE BUT WHO DID NOT DEVELOP CLINICAL SERUM SICKNESS; FROM PATIENT 14, WHO WAS ASTHMATIC AND HAD AN IMMEDIATE REACTION; FROM PATIENT 15, WHO HAD SERUM SICKNESS AFTER TREATMENT WITH PEPSIN-DIGESTED SCARLET-FEVER ANTITOXIN, AND PATIENT 16, WHO HAD SERUM SICKNESS WITHOUT A RASH

	Serum sickness	Antihorse precipitin titer	Heterophile agglutinative titer non abs.*	Produces passive serum reaction				Inverse passive transfer reaction P.K. technique
				general		local		
				pos.	neg.	pos.	neg.	
11	0	0	1/128			2	0	—
12	0	0	1/32			1	1	—
13	0	1/100	1/32			2	0	—
14	+	0	1/10	1	0	4	0	+
15	+	0	1/80			2	0	+
16	+	1/100	1/160			2	0	+

\* Guinea pig and beef R.B.C. absorption not done.

Symbols: —, not done; +, positive; 0, negative; P.K., Prausnitz-Kuestner.

to horse serum or horse dander was not known previously. Serum prepared from his blood, drawn 10 days after this episode, gave a negative reaction for precipitin and only a low titer (1/10 unabsorbed for the heterophile agglutinative antibody). Yet, this serum reproduced both the local and general forms of passive serum sickness and contained the passive transfer antibody for horse serum.

*Effect of Benadryl on Antihorse-Antibody Formation.* Patient #15 had serum sickness following treatment with refined diphtheria antitoxin, 20,000 units intravenously and 20,000 units intramuscularly. The serum sickness was treated with Benadryl (50 milligrams, 4 times daily) with what seemed to be a favorable effect on the itching and urticaria. Blood was drawn 72 hours after medication was discontinued and 6 days later, all clinical evidence of serum sickness had disappeared. No precipitin was demonstrated, but the serum did produce the local form of passive serum sickness.

*Antibody Formation Following Serum Sickness without a Rash.* Serum



#16 was obtained from a child who was treated with 5 ml. of scarlet-fever antitoxin. After 7 days, he ran an unexplained temperature which persisted for several days. Serum sickness was suspected, although no rash appeared. On the 14th day after treatment with antitoxin, his blood was drawn. It revealed the presence of precipitin, a heterophile antibody, and ability to produce the local passive serum reaction, the latter even after the serum was heated at 60°C. for one hour, thus establishing the thermostability of this serum sickness antibody. In accordance with the findings of Loveless, this antibody lacks the thermolabile quality of a reagin. Since this is a single observation, confirmation is necessary before thermostability of the serum sickness antibody can be concluded.

### Discussion

The data of analysis of the antibody determinations permit certain deductions as to the nature of the serum sickness antibody. The presence of this antibody would seem to be established if a particular serum is able to produce passive serum sickness in a horse serum-treated patient. Passively-induced serum sickness, the local serum reaction demonstrated by the Voss technique, the passive transfer reaction demonstrated by the Prausnitz-Kuestner technique, and the skin reaction of horse serum-treated patients to intradermal injections of antihorse antibody-containing serum are all similar reactions except in degree. Therefore, it may be concluded that a positive reaction demonstrated by any one of these techniques indicates the presence of the serum sickness antibody.

Using these criteria for the demonstration of the serum sickness antibody, it was established that it occurs in most patients convalescing from serum sickness, that it may be found in horse serum-treated individuals who do not develop clinical manifestations of serum sickness, and that it may appear in patients who are treated with normal horse serum. It was also shown that the serum sickness antibody was present in the blood of a horse serum-sensitive patient 10 days after a severe immediate reaction to horse serum.

The serum sickness antibody seem to have no constant relationship to the heterophile antibody. This observation is in keeping with the demonstration by Powell *et al.*<sup>20</sup> that horse serum from which the heterophile antigen had been removed was still productive of serum sickness. Likewise, the serum sickness antibody and the anaphylactic antibody did not invariably occur simultaneously and seem to be distinct. One of the 6 sera which produced anaphylaxis in guinea pigs failed to induce passive serum sickness.

Finally, the data show that the presence of precipitin seems to have been unrelated to the presence of the antibody responsible for production of the passive serum sickness reactions. Of the 16 sera tested, only one had no precipitin and failed to induce passive serum sickness, while 5 of the 16 contained no demonstrable precipitin but did possess the ability to induce passive serum sickness reactions. Of the 13 samples of S.S.C.S., 4 had no demonstrable precipitin, yet 3 of the 4 sera did induce passive serum sickness reactions. Of the 3 sera obtained from horse serum-treated patients who had no clinical evidence of serum sickness, only 1 contained precipitin, but all 3 produced the localized form of serum sickness. The most important

observations against the views expressed by Hamburger and Moro,<sup>2</sup> Longcope and Rackeman,<sup>21</sup> Mackenzie and Leake,<sup>22</sup> and others—namely, that precipitin and the serum sickness antibody are identical—are those made with S.S.C.S. #10. This serum had a high titer for antihorse precipitin. It produced anaphylaxis in guinea pigs but failed consistently to induce any of the reactions ascribed to the serum sickness antibody.

These data corroborate the original views expressed by von Pirquet and Schick, namely, that serum sickness is the result of an antigen-antibody reaction and that the serum sickness antibody is distinct from precipitin.

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## CONTACT ALLERGY OF THE SKIN

By Max Grolnick

*Jewish Hospital, Brooklyn, New York*

After definition and identification of the subject, this presentation will be primarily concerned with the mechanism or manner of the development of contact allergy. An attempt will be made to present such pertinent experimental data from the available medical literature as may aid in the formulation of a logical hypothesis for such a mechanism. The introduction of a vast literature on the clinical aspects of contact dermatitis will be avoided, except in particular instances where the experimental data suggest some clinical implication. Thus, many excellent clinical presentations by the later students of contact allergy, such as Schwartz, Sulzberger, Downing, Stokes, Shelmire, Epstein, and others will be omitted. Highly controversial aspects of the subject will not be entered into.

Contact allergy is a manifestation in the skin of an inflammatory reaction, in response to an acquired hypersensitiveness to previous contact with an effective, specific, sensitizing substance. It can be reproduced at will by topical contact with the same or a related allergenic or sensitizing material. Clinically, it is represented by a superficial inflammation of the skin, generally with vesiculation, fairly sharply delineated, appearing in acute or chronic form and tending to recur. The specific diagnostic reaction, the patch test, is of the delayed and not the immediate whealing type, occurring in the already sensitized subject usually after twenty-four hours of suitable surface contact. It may appear after contact for a few hours or several days. There is no evidence of an inherited predisposition, a characteristic distinguishing it from the skin manifestations commonly referred to as atopic eczema or dermatitis of infancy or neurodermatitis of early childhood and early adult life.

Contact dermatitis is commonly encountered in civilian medical practice and may be caused by a variety of substances such as poison ivy and other similar plants, by household materials, cosmetics, clothing dyes, adhesive plaster, and others. Injudicious and continued use of some local therapeutic agents may cause prolongation of the original lesion by inducing new sensitivities. Contact allergy is of special import among the various dermatoses encountered in industry, particularly since the advent of the newer synthetic products. Bonnevie,<sup>1</sup> in a discussion of the occupational dermatoses, stated that eczema (contact dermatitis) was a disease of civilization, having become more prevalent with industrial expansion.

Contact allergy involving the skin is variously referred to as eczema ("Ekzem" of the European school), eczematous contact-type dermatitis (Sulzberger), dermatitis venenata, or epidermitis (S. Epstein). In this presentation, the general clinical entity just identified will be referred to for the most part as contact dermatitis, though the term eczema may be used at times in referring to the older literature.

Josef Jadassohn<sup>2</sup> was the first to report on untoward (allergic) reactions

in the skin due to certain drugs administered internally or parenterally. He made observations on the clinical phenomena which were involved in the causation of eczema and referred to such factors as sensitization, incubation period, and method of spreading of the sensitization. He also presented his concept regarding the ability of the skin to react by means of the surface application to the skin of the suspected agent. This was referred to as the "funktioneller Hautprüfung" or functional test of the skin, now commonly called the patch test. Several modifications of the original technique are in general usage. Bloch<sup>3</sup> employed the term "eczema test" and specified five degrees of reaction ranging from erythema to tissue necrosis.

### *Histopathology*

According to Bloch and Steiner-Wourlish,<sup>4</sup> the pathologic process in human eczema affected most strongly the cells of the epidermis and the underlying layer of the dermis. In the milder reactions, the participation of the connective tissue was slight. The authors felt that the involvement of the epidermis was characteristic of eczema and served to differentiate it histologically from simple inflammatory reactions. Sulzberger<sup>5</sup> stated that the predominating shock organ in contact dermatitis was the epidermis and that the cutis participated to a lesser degree, if at all. The initial lesion was intraepidermal edema, known histologically as spongiosis. Following this, the lesion was evident as intraepidermal vesicles, which when fully developed were visible clinically as blebs of varying size, some of which oozed readily. In a comparative histologic study of the various eczematoid dermatoses, however, Sachs, Miller, and Gray<sup>6</sup> detected little intercellular edema or spongiosis about the epidermic vesicles and little evidence of edema in the rest of the epidermis. In the upper cutis and sub-epidermic zone, there were dilatation of the vessels and some interstitial edema. In addition, there was found here a moderately diffuse cellular infiltration of small round cells and wandering connective tissue cells. Rokstad<sup>7</sup> felt that histologic investigation could hardly determine whether the epithelial changes were primary or secondary to the alterations in the blood vessels in the corium. The writer has observed a number of times that the first visible evidence in the development of the spontaneous flare-up reaction was a petechial-like eruption at the site of application of the sensitizing agent, and that this was present even before there was palpable edema at the site. It would appear then, from a consideration of these reports, that, in addition to the part played by the epidermis, the structures in the papillary and upper cutis zones are involved in the pathology of contact allergy of the skin.

The histology of the specific lesion of contact dermatitis would appear to differ in no wise from that of the non-specific one. Miescher<sup>8</sup> reported the histologic response in the sensitive individual to be the same as that induced by the application to the skin of primary irritants such as croton oil, turpentine, cantharides, and others. Mom and Noussitou<sup>9</sup> demonstrated similar findings at both the actively sensitized specific site (dinitrochloro-benzene) and at that produced by an irritating concentrated solution of the same

excitant. The skin showed initial intraepidermal spongiosis and vesicle formation, and in the later stages there was lymphatic perivascular infiltration of the vessels of the papillary body and superficial dermis.

In a comparison of the specific reaction in man with that in the guinea pig, produced by painting the skin with ragweed oil or 2-4 dinitrochlorobenzene, Ginsberg, Becker, and Becker<sup>10</sup> and Ginsberg, Stewart, and Becker<sup>11</sup> demonstrated considerable histologic difference. In the animal, there was a slight intracellular and intercellular edema, but definite spongiosis was not seen. Vesicle formation was lacking. The cutis showed diffuse edema and round-cell infiltration. In man, however, the edema tended to be localized in the epidermis, where it led to vesicle formation, and in the cutis round cell infiltration was predominantly perivascular. The authors felt that these differences were to be explained not by a disparity in the degree of reaction but, rather, by the dissimilarity in structure of animal and human skin.

In evaluating some of the experimental material to be presented hereinafter, it is essential to bear in mind the apparent similarity between the specific and non-specific cutaneous reaction, and the lack of similarity between the animal and human cutaneous response.

### *Mechanism of Development of Contact Dermatitis*

In considering the role played by allergy and immunology in the mechanism of contact allergy of the skin, it is well to look back to a presentation by Coca<sup>12</sup> in 1926 on the relation of human hypersensitiveness to anaphylaxis. He stated that the task of confining his paper to studies having some bearing on the conditions of hypersensitiveness affecting human beings was "difficult because nearly every question to be presented is in active controversy." In fact, very little space was devoted to any discussion of the subject of contact dermatitis. Certainly, in the past two decades, there has been a most significant advance in the understanding of the various agencies which affect the development of skin sensitization of this type.

The subject of mechanism, which will constitute the major part of this paper, will be presented under four general divisions, namely: (1) factors involved in active sensitization; (2) manner of spread of sensitization; (3) immunology; and (4) chemical hypersensitiveness.

(1) FACTORS IN SENSITIZATION. The requisites for inducing sensitization of the skin are a suitable sensitizing agent, an experimental subject which is capable of responding in an allergic manner to exposure to this substance, and a method of contact between subject and allergenic substance which will stimulate the immunologic processes.

*The Sensitizing Substance.* Bloch<sup>4</sup> stressed the importance of a specifically active substance. Thus, though poison ivy, primula extract, and formalin were able to sensitize large numbers of subjects, camomile accomplished this infrequently. The concentration of the excitant was likewise of significance. When the human skin was treated by a dilute preparation of the primulin plant, only 42 per cent of the subjects were sensitized. The use of a concentrated extract was effective in sensitizing all the subjects. The

strength of the excitant also influenced the time of appearance of the sensitization. Thus, when Silverberg<sup>13</sup> treated the same area of skin in humans with a daily unction of a 10 per cent ointment of mesotan (salicylic ester), the incubation period was 20 to 25 days. When a 100 per cent ointment was employed, sensitivity occurred in 7 to 10 days.

It was stated by Stauffer<sup>14</sup> and others that a substance, to be a good sensitizing agent, must be a primary irritant when used in high concentration. Simon, Simon, Rackemann, and Dienes<sup>15</sup> observed that the skin of guinea pigs always reacted to the first application of poison-ivy extract with redness and swelling of the skin. Wedroff and Dolgoff<sup>16</sup> noted the primary irritation caused by a strong solution of 2-4 dinitrochlorobenzene. Landsteiner and Jacobs<sup>17</sup> asserted that most substances which were capable of sensitizing were in themselves irritating, though such an effective excitant as paraphenylene-diamine had little primary action on the skin, and some irritating chemicals were incapable of inducing sensitization. Straus,<sup>18</sup> on the other hand, was unable to find any irritation on the skin of newborn infants, at sites treated with a strong poison-ivy paste, or on the skin of the Rhesus monkey<sup>19</sup> treated with an undiluted poison-ivy oil for several days.

Clinically, the frequent establishment of sensitivity, after apparent healing of a vesicated skin, induced by direct contact with irritant chemicals used in the manufacture of poison gases was observed by Goldblatt.<sup>20</sup> The likeness in gross appearance between the specific and non-specific lesions of contact dermatitis was observed by Bonnevie<sup>1</sup> and others, and the similarity in histologic picture was referred to previously.<sup>8, 9</sup>

*Exposure to Allergen.* The necessity of exposure to the excitant has been established as a requisite in the development of sensitivity. Spain<sup>21</sup> demonstrated the absence of any reactions to poison-ivy extract in 18 infants from 5 weeks to 18 months of age. Reactions, however, were obtained in a group of children past the age of 8 years and in adults. On an expedition to the Baffin Islands, Heinbecker<sup>22</sup> was unable to elicit any skin response to tests with concentrated poison-ivy extract in 65 normal Eskimos. Likewise, Straus<sup>18</sup> found that 119 newborn infants failed to react to the first patch test with poison-ivy extract. The application to the skin of a paste made of equal parts of lanolin and ivy residue for a 6- to 8-day period did not produce any skin response, but, when the infants were tested from 2 to 4 weeks later, there was an incidence of positive reactions of 73 per cent. Among the subjects tested by Grolnick<sup>23</sup> to determine the existence of sensitivity to krameria, there were 26 children, ranging from 8 months to 13 years of age. No positive reactions were obtained, but four of the children became sensitized by the 48-hour test, after an incubation period of from 8 to 15 days.

*Technique of Sensitization.* The methods used in active sensitization of experimental subjects employed, for the most part, some means of surface contact of the sensitizing substance with the skin. There were some variations in the general technique. Low<sup>24</sup> sensitized himself and one other to primula extract by treating the skin from which the horny layer had been removed. The same method applied to 6 others was ineffective. Bloch

and Steiner-Wourlish<sup>4</sup> rubbed primula extract into the intact skin or into sites traumatized with "glass paper." When necessary, this procedure was repeated up to 10 times, utilizing various locations on the body. The application of a concentrated extract succeeded in sensitizing all of 12 human subjects after 1 to 3 treatments, in most instances after the first. Schwarzschild<sup>25</sup> sensitized his subjects by applying orthoform daily to the same area of skin and covering the site after each treatment with an adhesive plaster patch. Muller<sup>26</sup> employed the same technique in sensitizing humans with a 2 per cent ursol (paraphenylenediamine) ointment applied on either the intact or abraded skin. Newborn infants were sensitized by Straus<sup>18, 27</sup> by means of a single prolonged patch-test application of a strong poison-ivy extract.

Wedroff and Dolgoff<sup>16</sup> introduced the drop-method technique in their experiments in active sensitization of humans. They succeeded in rendering sensitive 70 per cent of their subjects with a drop of a 10 per cent solution of dinitrochlorobenzene. This method has since been used extensively for experimental sensitization of animals or humans.

Straus<sup>19</sup> applied the undiluted poison-ivy oil as a patch test to the skin of the Rhesus monkey for 48 hours without initial response. A similar test made 7 to 10 days later yielded a typical contact reaction. Grolnick,<sup>23</sup> in sensitizing human subjects to krameria, a plant extractive, employed the technique of repeated patch-test applications of the excitant for 1 or 2 days at spaced weekly intervals. If sensitization failed to occur after 8 such treatments, a final patch test was applied for a 7-day period. Of 37 subjects studied by this method, 86 per cent were actively sensitized. In unpublished experiments, it was found that an initial 7-day treatment yielded an even higher percentage of successful results, most of them occurring after the initial application. These techniques enabled the writer to make a critical study of the flare-up reaction.

In summary, active sensitization of the skin of experimental subjects was readily induced by surface treatment of the intact or traumatized skin, with a concentrated preparation of a potent excitant, either for a prolonged period or for shorter repeated periods of time. The various authors reported that sensitivity was generalized, as demonstrated by positive tests on any part of the integument.

Methods of rendering the skin sensitive by means other than surface application were also attempted. Frei<sup>28</sup> and Sulzberger<sup>29</sup> were able to sensitize the guinea pig by the intracutaneous administration of neoarsphenamine. They determined the existence of tissue sensitivity by means of an *intracutaneous test*. Sulzberger could not demonstrate sensitivity by similar test in animals treated by the intracardiac, intrapulmonary, intraperitoneal, intravenous, or intramuscular routes of administration. Simon<sup>30</sup> failed to sensitize guinea pigs to poison ivy by the latter three channels, yet succeeded by the direct application of the excitant to the skin. Straus<sup>27</sup> fed 10 newborn infants an alcoholic solution of poison-ivy extract, but could not elicit a positive test with the extract several weeks later. Nor was he able to sensitize infants by the subcutaneous injection of a similar extract.

Haxthausen<sup>31</sup> reported sensitization of the skin of humans with dinitrochlorobenzene introduced intracutaneously or subcutaneously. No other investigator has confirmed sensitization in humans by this procedure. Sensitization by the intramuscular route was achieved only when horse or human serum was added to the chemical. It was shown by Ginsberg, Stewart, and Becker<sup>11</sup> that guinea pigs sensitized by surface application of ragweed oil or poison-ivy extract attained the same degree of sensitization as those sensitized by its intracutaneous injection. But the authors claimed that contact tests with graded dilutions of the antigen were much easier to interpret than were the intracutaneous tests. Landsteiner and Chase<sup>32</sup> were able to demonstrate skin sensitivity in guinea pigs given intraperitoneal injections of picryl chloride or of conjugates of the latter and homologous red cell stromata, after preliminary treatment of the animals with tubercle bacilli. A high degree of skin sensitiveness was determined by the surface application of a dilute solution of picryl chloride. More recently, Strauss and Spain<sup>33</sup> reported the occurrence of contact-type reactions with poison-ivy extract in guinea pigs treated previously by intraperitoneal injections of aqueous alum-precipitated poison-ivy extract.

The view is held by some that contact-type dermatitis can occur clinically from influences other than external. Block<sup>3</sup> claimed that the eczematous reaction could arise experimentally by the ingestion or injection of iodine, quinine, resorcin, and arsphenamine in sensitive individuals. Horsfall<sup>34</sup> induced a characteristic exacerbation of dermatitis of the hands in a patient sensitive by contact to 1:8 million solution formaldehyde within 15½ hours of inhaling the vapor in a dilution of 1:95,000. Adequate precaution to exclude direct contact of the vapor with the skin had apparently been taken. Fisher<sup>35</sup> reported the lighting-up of previously healed areas of sulfonamide (contact) dermatitis following the oral ingestion of the specific drug. And, finally, the eruptions which occur not uncommonly in ivy-sensitive individuals during oral desensitization treatment are well known. It is not always clear from the descriptions of the skin manifestations induced by these various procedures whether they are identical with typical contact dermatitis. Furthermore, it should be recognized that *these skin responses generally occurred in the already sensitive individual.*

*The Experimental Subject.* Many animals have been employed in the study of experimental contact allergy, but the guinea pig has been the most widely used. A critical viewpoint, however, is needed in correlating the findings in animals with those in humans. Von Adelung<sup>36</sup> sensitized the rabbit to rhus toxin and the leaves, though most later observers did not find the rabbit to be suitable for study. Stewart and Cormia<sup>37</sup> were able to sensitize guinea pigs by repeated applications of nickel salts to the same skin area but failed to do so by intracutaneous injections with the same substances. These authors stated that Walthard succeeded in sensitizing by surface treatment of the skin with nickel but that Coca and Milford failed to confirm these findings.

Dienes<sup>38</sup> referred to the production of skin sensitization in the guinea pig with a number of chemical substances known to cause industrial eczema:



diphenylamin, phenylhydrazine, nickel, salvarsan, and the plant excitants, primula and poison ivy. Rabbits treated under the same conditions failed to respond. Brunsting and Bailey<sup>39</sup> sensitized 3 of 14 guinea pigs to the oily extract of the ragweed plant, but Kile<sup>40</sup> was unsuccessful with the plant oils of giant ragweed, sage, or orris root.

Straus<sup>19</sup> did not feel that the guinea pig, rabbit, or white rat were satisfactory for the study of sensitization to poison ivy, inasmuch as there was, at times, a marked initial irritative response. The Rhesus monkey did not present such a disadvantage. The fully developed mild reaction in this animal exhibited a sharply demarcated erythematous elevated area with superimposed follicular papules which oozed or bled easily. Scaling, which followed, lasted for a few days. The marked reaction consisted of erythema, elevation, central vesicobullae, and surrounding follicular papules. The picture was similar to that which occurred in humans. This description was in sharp contrast to the deep inflammatory reactions occurring in the guinea pig.

Grolnick<sup>41</sup> failed to sensitize the skin of the chicken with krameria by the method of successive applications of the excitant at spaced intervals. When poison ivy was employed as the excitant, sensitization was induced in 5 of 8 birds and was questionable in 1. The appearance of the reaction in successive stages was as follows: an initial erythema followed by small punctate hemorrhages; then an elevation of the follicles of the skin, rendering them palpable. Next, there was a faint edema represented by a palpable thickening of the skin, and this was followed by visible edema and vesicle formation. The vesicles were extremely fragile, so that the skin was readily denuded, with consequent oozing and crusting. The crusts were pale yellow at first (from admixture with the serous fluid) and later became dark. The reactions were fairly well limited to the sites of application of the excitant. These responses had been observed by H. Straus, who commented on their close resemblance to those elicited in the sensitized monkey.

Landsteiner and DiSomma<sup>42</sup> claimed sensitization of 60 per cent of 38 guinea pigs by repeated surface applications with diazomethane, a non-aromatic substance known to cause hay fever and asthma in lab workers. Description of the lesions suggested an irritative type of reaction. These authors also reported sensitization of two or three hogs to mustard oil by the same method and failures in guinea pigs, rabbits, and monkeys. The skin of the duck was found by Mirsky and Goldman<sup>43</sup> to be satisfactory for the production of bullae with various skin irritants such as croton oil, formaldehyde, and mustard oil. Microscopically the fluid was found to be subepidermal, the epidermis remaining intact.

Thus, from an extensive review of the role of the experimental animal, it would appear that the response in the monkey most closely resembled the human lesion, and that, while some findings obtained from guinea pig studies are applicable to man, others cannot be so correlated. It might also seem worthwhile to explore the possibility of further study with the fowl.

The human subject lends itself to investigations of the problem of contact allergy. Horsfall<sup>34</sup> did not believe that the erythematous papules ob-

tained by intracutaneous testing of a markedly formaldehyde-sensitive patient were entirely specific. These reactions resembled those described by Landsteiner and his co-workers in sensitized guinea pigs. Wedroff and Dolgoff<sup>16</sup> preferred humans in their studies, for they believed that the findings in experimental animals were not applicable to man. Sulzberger and Baer<sup>44</sup> and Sulzberger and Rostenberg<sup>45</sup> stated that the contact type of reaction in the human skin was not identical with the skin sensitivity demonstrated in the guinea pig by means of the intracutaneous test or even by surface application of the excitant. Furthermore, the human subject presented an advantage, in that the experimental approach paralleled the manner of clinical exposure, and the reaction was the same clinically and histologically as the disease. Haxthausen<sup>46, 47</sup> employed various surgical procedures in his studies on the manner of spread of sensitivity in the human subject. Later, considerations in the mechanism of evolution of contact allergy will point out the advantages of the use of the human as an experimental subject.

*The Flare-Up Reaction.* This phenomenon is the first visible evidence that a subject previously non-sensitive has developed a state of hypersensitivity. The term was used by Frei<sup>48</sup> to describe the spontaneous appearance (*Aufflammungsphänomen*), in the human, of deep inflammatory reactions at the sites of intracutaneous tests with neosalvarsan introduced 11 to 12 days previously. The flare-up reaction was likewise observed after a 5 to 6 day interval in guinea pigs treated with the same drug.<sup>28</sup> Sulzberger<sup>29</sup> confirmed the findings in the guinea pig. The authors explained this change as the reaction between the then hypersensitive cells and the sensitizing substance remaining at the site of the injection. Dienes and Simon<sup>49</sup> described flare-up reactions in guinea pigs at the sites of intracutaneous tests with human serum given 5-6 days prior. By using several antigenic substances, a variation in their ability to produce flare-up reactions was found, turtle egg being effective in nearly all animals, human serum in only one of four. Egg white and horse serum were capable of producing hypersensitivity but not spontaneous flares. Reactions of a similar nature to bacteria were demonstrated by Andrewes, Derick, and Swift<sup>50</sup> and by others.

The spontaneous flare-up as a manifestation of acquired hypersensitivity of the skin (contact allergy) has been reported by most investigators either by name or description. The term should be used only where the activation of a previously inactive site is implied. The phenomenon occurs at the culmination of the incubation period and indicates the advent of a state of hypersensitivity. It probably signifies an interaction between hypersensitive skin cells and an allergenic substance. The term should not be employed to indicate the lighting-up of the healed site of a specific test or a clinical dermatitis which may follow the appearance of a positive specific patch or surface test.

Bloch and Steiner-Wourlich<sup>4</sup> discussed the lighting-up of old, totally or partially healed sensitizing sites (*Impfherde*). They were probably dealing with what is now considered to be the spontaneous flare-up, inasmuch as the skin areas treated with primula extract had frequently been traumatized,

and, for this reason, the authors referred to healed sites. Schwarzschild<sup>25</sup> likewise referred to the activation of sensitizing sites in his experiments with orthoform. Wedroff and Dolgoff<sup>16</sup> described the flare-up reaction at the site of application of a drop of dinitrochlorobenzene after an interval of 8 to 24 days. At first, there was an initial erythema which was soon followed by a typical allergic contact-type reaction. Sulzberger and Rostenberg<sup>48</sup> observed flare-up of the sensitizing sites in over 50 per cent of their subjects. This occurred from 7 to 20 days after application of a drop of p-nitrosodimethylanilin and 2-4 dinitrochlorobenzene.

Grolnick<sup>28, 51</sup> undertook a systematic study of the flare-up reaction in humans sensitized with krameria. Eighteen subjects exhibited a typical flare-up response from 8 to 21 days following a single patch-test application with the excitants. Nineteen subjects were sensitized with from 2 to 5 successive applications of the allergen at weekly intervals. In these subjects, the appearance of a response, the flare-up reaction, at the final site of treatment was followed in 15 subjects by a spontaneous flare-up of the site of the preceding application, which up to that time had remained unchanged. In four additional subjects, flare-up occurred at two preceding and previously negative sites of application. These sites, moreover, were affected in the reverse order of their treatment and reacted in the majority of instances with less intensity than the initial flare-up reaction. The interval which elapsed between the time of treatment of these late responding areas and the appearance of the spontaneous reactions at these sites was from 10 to 43 days. Thus, the allergenic or antigenic substance had remained and apparently could stay fixed in the skin cells for as long as 43 days.

Another phase of this study was the determination of the minimal threshold of sensitivity to krameria, *i.e.*, how much of the excitant was available in the skin at the time of the spontaneous response and was therefore needed to evoke a reaction. Involved in this question, too, was the relation of the reverse order of flare-up to the concentration of the excitant at the respective skin sites. Thus, in further studies, patch-test applications were made horizontally on one arm, one or more inches apart, with an undiluted solution of the excitant, with 1:10, and with 1:100 dilutions. The patches were removed after 24 hours. The subject was then actively sensitized by making one or more applications of the undiluted excitant to the opposite extremity. The order of flare-up of the sites to which the graded dilutions of the excitant had been applied was then observed. Finally, as soon as flare-up occurred at the site to which the weakest dilution of the excitant had been applied, the subject was patch-tested with the excitant in dilutions of 1:1000, 1:10,000, and 1:100,000 in order to determine the level of sensitivity at this particular stage. The following findings were observed: The site to which undiluted excitant had been applied responded first in all six subjects. The site treated with 1:100 dilution always flared last. In 3 of the subjects, the level of sensitivity was demonstrated by a weak reaction at the site of the 1:100,000 dilution, in 2 by stronger reactions at the site of 1:10,000 dilution, and in 1 by a reaction with the 1:1000 solution. Applying these results to the findings of reverse order of flare-up reactions in

diminished intensity, it is apparent that the sites treated first had lost most of the excitant and that their reactivity had diminished during the inactive stage so that it was equivalent to that elicited by a solution many thousand times weaker than the original excitant.

*The Incubation Period.* As it relates to the experimental subject, the incubation period represents the time interval which elapses between the initial contact with an allergenic substance in a non-sensitive subject and the first appearance of a specific response at the site of exposure. Obviously then, the flare-up reaction signifies the culmination of those immunologic processes which have been stimulated by the primary exposure during this period. Where a single simple contact on a small area was effective in sensitizing the subject, there has been found a striking uniformity in the limits of the incubation period, namely from 7 to 24 days, regardless of the allergenic substance employed or its manner of application. Bloch and Steiner-Wourlich<sup>4</sup> found the incubation period for sensitization of the guinea pig with primula extract by simple inunction to be 7 to 10 days. By the drop method in humans, Wedroff and Dolgoff<sup>16</sup> demonstrated a range of 8 to 24 days for dinitrochlorobenzene. With the same procedure, Sulzberger and Rostenberg<sup>46</sup> induced sensitiveness to dinitrochlorobenzene or to p-nitrosodimethylanilin in from 7 to 20 days. Straus<sup>19</sup> reported the onset of sensitivity in the monkey 7 to 10 days following the simple patch-test application with poison-ivy extract. Grolnick<sup>28</sup> found the incubation period for sensitizing humans with krameria by means of a single patch-test exposure to be 8 to 21 days.

When sensitization procedures other than a single contact were employed, the range in incubation period became less uniform. By making daily applications with orthoform, Schwarzschild<sup>25</sup> sensitized humans in from 10 to 71 days. Silverberg<sup>13</sup> found an incubation period of from 20 to 25 days for sensitization to mesotan by daily inunction with a 10 per cent ointment. When a 100 per cent paste was used, the interval was reduced to 7 to 10 days.

Occasionally, a shorter period than seven days has been reported. Thus, Milford,<sup>52</sup> in testing a group of ragweed-sensitive hay fever patients by the intracutaneous method with a suspension of ragweed-pollen oil in 1 per cent alcoholic solution, noted the development in some cases of a severe dermatitis at the sites of the tests in from 5 to 21 days. It would appear that these were instances of sensitization by surface contact with the allergenic oil.

Most observers reported the change in response of the skin which occurs once sensitization has been effected. Reactions then appeared to suitable contact tests generally in from 24 to 48 hours, and not infrequently within the first 24 hours. Thus, the reaction time must be distinguished from the incubation period (Sulzberger<sup>4</sup>).

*Sensitization by Patch Test.* While this topic primarily carries clinical implications, it is entered into at this point because of erroneous conclusions presented by a number of otherwise careful investigators on the incidence of sensitivity to poison ivy. By patch-test studies with poison-ivy extracts

in various groups of subjects, an incidence of positive reactions of from 49 to 76 per cent was reported: Spain, 65 per cent;<sup>21</sup> Deibert, Menger, and Wigglesworth, 59 per cent;<sup>58</sup> Spain, Newell, and Meeker, 75 per cent;<sup>54</sup> Knowles, Decker, Pratt, and Clark, 49 per cent;<sup>55</sup> and Keeney, Sunday, Gay, and Lynch, 70 per cent.<sup>56</sup> A detailed consideration of each report would be too lengthy at this point, though analysis of several of these studies was made in a previous publication.<sup>51</sup> All of these investigations are subject to the same criticism, namely, that a highly concentrated extract of poison ivy, or the actual leaf itself, had been used and the period of application of the patch tests was for 2 to 7 days. It seems certain that many of the subjects had been actively sensitized, as can be ascertained not merely from a study of the available tables but from such statements by the authors as "reactions were noted after as long an interval following application of the patch test as 27 days," or, "reactions were observed from 20 to 228 hours after the test." One should then interpret the values of 49 to 76 per cent not only to include the incidence of sensitivity to poison ivy but, in addition, to denote to what extent sensitization could be induced with this excitant by the technique employed, *i.e.*, the susceptibility of humans to this allergen.

Further analysis of the report by Spain, Newell, and Meeker revealed that successive applications of the excitant had been made in increasing concentration, thus adding an additional factor which aided active sensitization. In a consideration of the manner of development of sensitization by a single or by repeated patch-test applications with the excitant, it was stated by the writer<sup>51</sup> that each successive stimulus to the skin with the excitant influenced the immunological processes which were developing, and that the final outcome or state of hypersensitiveness was the result of a summation of the individual stimuli. Certain clinical inferences may be drawn from the above findings, namely, that, when patch tests are done as a diagnostic procedure, some active excitants in sufficiently high concentration may sensitize even with a single short exposure. Also, the not uncommon practice of repeating diagnostic patch tests with the same or related allergens when tests are negative or doubtful should be discouraged. It is not improbable that the repetition of some diagnostic patch tests by one or several clinicians may actively sensitize the patient to some of the substances being applied in the tests.

Another observation which pertains to the possible influence of the patch test is the statement by Wedroff and Dolgoff<sup>18</sup> that, when sensitivity which had been actively induced was in the stage of regression, repetition of tests could restimulate the sensitization process. Likewise, one should examine critically a statement made by Stauffer,<sup>14</sup> a careful clinical investigator, that, because a patch-test reaction did not appear in 1-2 days, it was not to be assumed that the test was negative. The author had observed cases in which it did not appear for 15 days and was then particularly intense. Downing<sup>57</sup> and Bechet<sup>58</sup> recognized the potential sensitizing feature of the patch test and for this reason the former opposed the pre-employment test.

**Susceptibility.** This term will be used to signify the extent to which

active sensitization can be induced under favorable circumstances in subjects not previously exposed to the specific excitant. It represents the organism's propensity for being sensitized. The susceptibility of humans to sensitization with various excitants has been referred to previously, namely, primula extract (100 per cent),<sup>4</sup> dinitrochlorobenzene (70 per cent),<sup>16</sup> poison ivy (73 per cent),<sup>18</sup> orthoform (45 per cent),<sup>25</sup> and krameria (87 per cent).<sup>23</sup>

The influence of a personal or family incidence of allergy (atopic mechanism) was studied by several observers. No such relationship was found. Brown, Milford, and Coca<sup>59</sup> showed that sensitivity to ragweed oil occurred with equal frequency in both atopic and non-atopic subjects. A similar observation was made by Grolnick<sup>23</sup> in his studies with krameria. Schwartz<sup>60</sup> found that a personal or family history of allergic diseases was not preponderantly present in those affected with industrial dermatitis. Knowles, Decker, Pratt, and Clark<sup>65</sup> determined by testing a group of 200 medical students with poison-ivy extract that there were no significant differences in the incidence of a personal or family history of allergy among those who reacted as compared with those who did not.

The possible transmission of skin sensitivity of the contact type from parent to offspring has been the subject of study by several observers. Kile and Pepple<sup>61</sup> showed that offspring of guinea pigs sensitized with poison-ivy extract before or during pregnancy were not themselves sensitive. Grolnick<sup>62</sup> failed to detect any sensitivity in the newborn infants of 7 mothers sensitized to krameria in the last 2 trimesters of pregnancy. In 4 of the parents, the reaction of sensitization was in an active phase when the infants were born, indicating a high degree of sensitivity in the mother at this stage. Chase<sup>63</sup> tested the offspring of guinea pigs who were highly sensitive to dinitrochlorobenzene or poison ivy with the specific excitant, but in no instance could transfer of sensitivity be observed. In contrast to this absence of transfer of contact type allergy, the regular transmission of anaphylactic hypersensitiveness produced by tuberculo-protein from mother guinea pigs to their offspring was demonstrated by Corner and Stoner.<sup>64</sup>

The presence of individual variations in susceptibility in humans has been emphasized by a number of investigators. Since the experimental animals varied even more in susceptibility according to species and within the species, the use of the human subject has been preferred by some for such studies. Wedroff and Dolgoff<sup>16</sup> were able to sensitize 50 of 72 subjects with eczema of various types with a 10 per cent solution of dinitrochlorobenzene, but in 20 normal persons it was more difficult to accomplish this, and frequently a 30 per cent solution had to be used. Sulzberger and Rostenberg<sup>45</sup> simultaneously sensitized both control subjects and groups of patients with healed or recent contact dermatitis with p-nitrosodimethylanilin and 2-4 dinitrochlorobenzene. Not only were there individual variations in accepting sensitization with each chemical, but, in addition, the subjects with recent or active contact dermatitis were more readily sensitized (91 per cent to one or the other) than the non-contact group (53 per cent). This

suggested that patients with recent or existing allergic dermatitis were more readily sensitized by exposures to other chemicals than were previously unexposed persons.

The individual variation in susceptibility is evident likewise by a study of the following findings by Grolnick.<sup>28, 51</sup> Of thirty-seven subjects sensitized by from 1 to 5 repeated applications of krameria at weekly intervals, 18 required one patch-test application, 8 needed two, 6 had three, 4 had four, and 1 required five such contacts. In the guinea pig, Ginsberg, Stewart, and Becker<sup>11</sup> demonstrated that animals sensitized to dinitrochlorobenzene showed no greater tendency to become sensitized subsequently to ragweed-plant oil than did previously unsensitized animals or horse serum-sensitized (skin) ones. Chase<sup>63</sup> was able to show a general tendency for guinea pigs to be sensitized to the same extent to two common excitants of contact dermatitis—poison ivy and dinitrochlorobenzene. In individual animals, the comparative degree of sensitization varied, however, for each chemical.

The differences in species in their responses to attempts at sensitization were discussed in part under the heading, "Experimental Subject." In addition, Landsteiner and DiSomma<sup>42</sup> were able to sensitize the hog with allylisothiocyanate (mustard oil) but failed to do so in the guinea pig, monkey, and rabbit. Grolnick sensitized humans readily with krameria, but was unable to do so in the monkey, an animal easily sensitized by Straus<sup>19</sup> with poison-ivy extract.

The influence of heredity on susceptibility to sensitization of the contact type was studied by Landsteiner and Chase<sup>65</sup> and Chase.<sup>63</sup> Guinea pigs were reared under controlled conditions. Colonies of animals which reacted to chemical sensitization to a high or low degree were established. The parents were mated within each group. With continued selection of parents who were high reactors, there was an increase in the number of offspring who were readily sensitized. Among the progeny of low reactors, the incidence of resistance to sensitization was increased, but the results were less uniform and there were none of high reactivity. The authors concluded that chemical sensitization of the contact type was influenced by heredity.

In summarizing the subject of susceptibility, it is evident that there are marked species differences, in addition to those differences found in individuals in the species. There is no agreement as to whether individuals previously sensitive are more susceptible to sensitization by another excitant. An atopic influence in contact dermatitis was not found by several observers. Finally, the work of Landsteiner and Chase showed that, by selective inbreeding, a strain which is highly susceptible to skin sensitization can be developed.

*Permeability of Sensitized Skin.* Some clinicians have maintained that cutaneous areas which had been the seat of contact dermatitis showed positive patch-test reactions with the specific excitant, whereas adjacent and previously uninvolved areas did not respond. It was assumed that these reactions were specific in character and indicated a heightened or localized

tissue hypersensitiveness at the affected areas. Thus, Bloch<sup>3</sup> stated that, in eczema, sensitivity varied regionally in intensity and that tests could be positive only in certain areas, particularly where the greatest contact with the offending substance had taken place. A state of local sensitivity was implied, a condition originally referred to as such by Jadassohn<sup>66</sup> in a case of odol "eczema." Stauffer<sup>14</sup> concurred in this viewpoint, yet in a contradiction in the same paper stated that it was possible to get stronger reactions on normal skin than at the site of a previously healed eczema, ascribing this to local desensitization. Other observers have followed more or less the same line of reasoning, *e.g.*, Strandberg,<sup>67</sup> Boström,<sup>68</sup> and Sulzberger.<sup>6</sup>

It has been the impression of the writer that such variations in reactivity of the skin can be ascribed to a change in permeability at the involved areas so that these sites are readily influenced by *non-specific* factors. Grolnick, Bowman, and Walzer<sup>69</sup> studied the state of responsiveness of the skin at a healed site of contact dermatitis. They observed that when an area of skin in an atopic individual had become specifically sensitized as a site of contact dermatitis and was allowed to heal, subsequent testing of these sites with the specific wheal-inducing atopens, such as ragweed or dander extracts, revealed an altered response to the latter tests. Wheal formation at the dermatitis sites was greater in most instances than at normal comparative sites in the same individual. The more intense the original dermatitis, the more pronounced was the tendency to increased wheal formation. Grolnick<sup>70</sup> then studied the response of healed sites of contact dermatitis to the subsequent application of a second and unrelated allergenic excitant. Reactions were elicited in 42 subjects who were sensitive to either krameria or poison ivy, but not to both. The sites were allowed to heal completely. After a 4- to 12-week interval, each site was retested with the other excitant. In most instances, control tests had also been made on uninvolved skin. In 14 of the subjects, a typical contact-type reaction was present at the sites which had been twice stimulated. In 10 of the subjects on whom controls had been done, these sites were negative. In 4 subjects, there had been no controls. One must infer from this experiment that, although the second response, to all appearances, seemed like a specific reaction, it could not be so interpreted, inasmuch as control tests had been negative. The role of non-specific stimulation of a specifically sensitive area will be taken up by the writer in another communication. It is felt that the findings just described are related to the subject of so-called local sensitivity.

(2) MANNER OF SPREAD OF SENSITIZATION. An explanation of the means by which sensitization of the skin becomes generalized following application to a small area of a suitable sensitizing agent has been sought by a number of investigators. One method of study was through isolation of the treated area by surgical or chemical means. Simon<sup>80</sup> applied poison-ivy extract to local areas of skin in 12 guinea pigs. These sites were excised at intervals varying from 1 hour to 4 days. Spread of sensitization was prevented if the excision was done less than 18 hours after such treatment. Landsteiner and Chase<sup>71</sup> confirmed these findings in a similar experiment, determining that sensitization became generalized if the treated area was removed later



than 8 to 12 hours following the sensitizing application. In another study in the guinea pig, Simon<sup>50</sup> destroyed a ring of skin around the middle of the animal by cauterization with concentrated nitric acid, thus separating the front and hind parts of the animal. The posterior half was treated with poison-ivy extracts and both parts tested 10 days later. Reactions occurred in both halves. The author assumed that the spread of sensitization was not confined to the epidermis, but occurred by way of the blood stream or lymphatic system.

Straus and Coca<sup>72</sup> severed the continuity of the skin in the Rhesus monkey by a circular incision in the upper third of the arm. Application of a strong poison-ivy extract to the forearm resulted in sensitization of the part distal to the incision, but generalized skin sensitivity could not be demonstrated. When the experiment was carried out in reverse, there was failure to sensitize the forearm. The authors concluded that spread of sensitization was attributable "probably to a diffusion of the oily excitant through the oily substances normally present in the skin." In essence, these findings were confirmed by Schreiber and Mueller<sup>73</sup> and Schreus.<sup>74</sup> Islands of skin, the size of a half dollar, were isolated on the backs of guinea pigs by the removal of a narrow strip of skin, the incisions penetrating down to the muscle and fascia. The islands were painted daily with a 5 per cent solution of dinitrochlorobenzene until an "eczematous" reaction appeared on the 7th day. Testing the remainder of the body with a dilute solution of the excitant failed to elicit any reaction. In another group of animals, the experiment was performed in reverse, reactions appearing on any part of the integument excepting the isolated skin islands.

Landsteiner and Chase<sup>71</sup> attempted to show that mere severance of the skin did not prevent the spread of sensitization to the entire skin surface unless the continuity of the superficial lymphatic vessels overlying the skin muscle (*panniculus carnosus*) was interrupted. When skin islands were isolated in the guinea pig and then treated by application with a strong poison-ivy extract, spread of sensitization was prevented almost uniformly, provided the incision included the skin muscle. The authors advanced a theory of sensitization, namely, that the chemical agent reacted quickly with tissue substance to form conjugates and these were transported by way of lymphatic vessels into the blood stream. They failed to explain why sensitization of the skin did not occur after the deposition of the excitant either subcutaneously or intramuscularly—findings which they and other investigators had ascertained.

Haxthausen<sup>46</sup> likewise claimed the demonstration of a hematogenous spread. Areas of skin in the epigastria of 3 human subjects were treated with dinitrochlorobenzene. The borders of the treated sites were then incised through the cutis, but not down to the fascia. The incisions were made at several intervals, at the time of treatment of the skin, 3 days, and 8 days later. Eventually, sensitivity of equal intensity was demonstrated both within and outside the incised areas. The author felt that the incisions might have been too superficial to prevent spread of sensitization. The same author<sup>47</sup> sensitized an area of skin in one of each of two pairs of twins. After intervals of 28 and 38 days, respectively, skin flaps were transplanted

from the sensitized to the non-sensitive twin in each pair, and conversely. Three weeks later, when healing was complete, tests with the specific excitant, dinitrochlorobenzene, were positive only in the two sensitive subjects, both over the general skin surface and on the previously non-sensitive skin flaps. Whereas the author interpreted these findings as evidence of some factor conveyed through the blood of the sensitized subject, one could just as readily draw the conclusion that the normal skin flap had been sensitized by diffusion from the adjacent hypersensitive skin. Thus, in the interpretation of the results of the aforementioned studies involving the continuity of the skin, there is definite variance in opinion.

Another method of study of the spread of sensitization entailed an alteration of the area of skin which was to be sensitized. Simon, Simon, Rackemann, and Dienes<sup>18</sup> produced injury of a skin site in the guinea pig by treatment with cowpox virus. With the appearance of the infection, the site was treated with poison-ivy extract in an attempt to sensitize the animal. Subsequent testing with poison-ivy extract in these animals and in sensitized control animals revealed a more striking sensitivity in the latter group. Haxthausen<sup>46</sup> was able to suppress sensitization of the human skin to dinitrochlorobenzene by treating an area with carbon dioxide snow as late as 8 days following application of the sensitizing chemical. In another experiment in the guinea pig, if selected skin sites were first treated with the freezing agent and then treated with dinitrochlorobenzene immediately thereafter, or after 2, 4, and 8 days, sensitization was suppressed in 4 of 5 animals in each group (total of 25 animals). Rokstad<sup>7</sup> was able to inhibit contact-type reactions by compression of the local areas. Thus, alteration of the skin by some means made it possible to influence sensitization. Confirmation of these experiments is lacking, however. Mom and Noussitou<sup>9</sup> could not prevent sensitization in humans with dinitrochlorobenzene by prior novocaine block of the nerve supply.

All of the experiments just cited were concerned with the initial phase of the development of sensitization, before the appearance of such evidences of sensitization as the spontaneous flare-up or the presence of a positive 24-hour patch-test reaction. The writer<sup>61</sup> inquired into this latter phase of the problem through further study of the flare-up phenomenon. By way of summary, human subjects had been sensitized by means of 1 to 5 successive patch-test applications of krameria extract at weekly intervals. Sensitivity was evident when a typical vesicular reaction appeared at the last treated site (flare-up). This was followed in numerous instances by the spontaneous appearance of responses at one or more preceding and previously negative sites of application. Moreover, flare-up of sites always occurred in the reverse order to that in which they had been treated. It was then shown that the order of flare-up was determined by the amount of fixed allergen which remained at the involved site, *i.e.*, the most recently treated site possessed the largest amount of excitant and was therefore the first to flare up after sensitivity had been effectuated. The oldest site, on the other hand, was the last to respond, since most of the excitant had been eliminated.

It was to be expected, then, that with the onset of sensitization sites ex-

posed simultaneously to the same allergen would flare up at the same time. Thus, in a further study, the excitant was applied simultaneously to two different parts of the body, *e.g.*, both arms, or both thighs, or one arm and one thigh. Following this, successive sensitizing applications with the excitant were made either close to or at a little distance from one of the two simultaneously treated areas. It was found that in the majority of instances the flare-up occurred earlier and the reaction was at first more intense at the area which was closest to the final sensitizing site than at the one more distant. One would have to infer from these findings that there had been a slow diffusion, by way of the skin, of some sensitizing factor, either allergen or a reacting substance. For, if spread of this factor had been entirely by the blood stream, one should expect sites treated simultaneously with the same concentration of excitant to flare up at the same time.

A point in confirmation of these findings is the observation by Haxthausen<sup>46</sup> that, when sensitization appeared at the "spontaneous" site, reactions were demonstrated first in the proximity of this area but, in 1 to 2 days, all areas reacted. Finally, from all of the foregoing experiments, regardless of technique employed or the outcome, the very significant role of some part of the skin in the ultimate establishment of a generalized skin sensitivity is apparent. The skin is not merely the end point, it is also the medium.

(3) **IMMUNOLOGY.** Though the subject of immunology in contact allergy of the skin is closely integrated with chemical hypersensitiveness, it will be discussed first, and separately from it, for the purpose of orderly presentation. Some overlapping will occur of necessity.

In human contact dermatitis, the diagnostic test is the patch, surface, contact, or percutaneous test. Its functions and characteristics can be enumerated as follows: (1) it represents an actual reproduction of the clinical lesion in a local area involving the tissue which is the seat of the disease; (2) substances which are readily available and easily applied are used in the tests instead of prepared, biological products; (3) the nature of the local response is the same generally, regardless of the substance employed to elicit the specific reaction; (4) the reaction is specific, though it may not be etiologic in significance; (5) reactions are generally multiple, and this may be due in part to sensitivities acquired by topical remedies used in the treatment of the clinical dermatitis; (6) repetition of patch tests may cause new sensitivities; (7) healed patch-test sites may light up on repetition of tests; (8) lighting-up of the clinical dermatitis may be brought about by specific reactions to patch tests.

Though contact dermatitis represents a delayed type of allergic response and is elicited by the contact or patch test, several references in the literature to the occurrence of the immediate wheal type of reaction will be recognized. Horsfall<sup>44</sup> obtained immediate wheal and erythema reactions by intracutaneous tests with formalized proteins (human, rabbit, or horse serum) in a patient with a formaldehyde dermatitis. Passive transfer of the test was not successful. Intracutaneous tests with formaldehyde yielded

erythematous papules of the delayed type similar to those described by Landsteiner in guinea pigs. Precipitin and complement fixation reactions were absent. Confirmation of these findings has not been reported. Landsteiner and Jacobs<sup>75</sup> obtained wheal responses to scratch tests with conjugates in guinea pigs sensitized with protein conjugates of acylchloride, but no reactions were elicited with the simple chemical itself. Jacobs, Golden, and Kelley<sup>76</sup> reported wheal reactions by scratch test in guinea pigs rendered sensitive to simple chemicals of the anhydride group, particularly to citraconic anhydride.

In interpreting the demonstration of wheal-type reactions, cognizance must be taken of the fact the typical contact dermatitis (except the Horsfall report) was not under consideration. The presence of wheal-type reactions to non-protein substances has been variously reported in drug sensitivity or in allergic mucous membrane manifestations: Kern,<sup>77</sup> phthalic anhydride; Mitrani,<sup>78</sup> thiamin chloride; Feinberg and Watrous,<sup>79</sup> chloramine T and halazone; and W. Sherman<sup>80</sup> and Whittemore and de Gara,<sup>81</sup> sulfadiazine. Such findings, though establishing the ability of simple non-protein chemicals to produce wheal-forming antibodies (and reagins), may not be used in explanation of an immunological mechanism in contact dermatitis.

Passive transfer of reactions to such known excitants of contact dermatitis as iodoform, paraphenylenediamine, mercury, primrose, and others have been reported in the past by Bruck, Klausner, Meyer, Bock, Mayer, Bieberstein, and others (quoted by Landsteiner and Jacobs<sup>17</sup>). Bloch,<sup>3</sup> however, was unable to demonstrate passive transfer with "eczematogenous" substances and cited Coca as having had the same experiences. In all recent reports in the literature, successful passive transfer of contact-type reactions has not been observed. Landsteiner and Chase<sup>87</sup> transferred an immediate wheal reaction to a non-sensitive guinea pig with serum from an animal sensitized systemically with citraconic anhydride. But, here again, the authors were not dealing with contact allergy.

Sulzberger and Katz<sup>83</sup> were unable to transfer any excitant from blister fluid obtained from blisters produced by specific (poison ivy) and non-specific irritants (mustard gas and lewisite). Pratt and Corson<sup>84</sup> likewise reported negative results with the contents of blister fluid from poison ivy-sensitive patients.

The possibility of passively sensitizing animals to simple chemicals was studied by Landsteiner and Chase.<sup>82</sup> Guinea pigs, previously made tuberculin-sensitive to enhance subsequent sensitization with chemicals, were readily sensitized by means of conjugates prepared from guinea pig red cell stromata and picryl-chloride. By injecting the peritoneal exudate from such animals into the peritoneum of normal guinea pigs, there developed in the latter a state of passive sensitization of the skin manifested by a positive reaction to the application on the skin of a solution of picryl-chloride in oil. The implications of this experiment are discussed under the next heading, "Chemical Hypersensitiveness."

Anaphylactic shock could not be induced by Muller<sup>26</sup> by means of an intracardiac injection of ursol in guinea pigs sensitized 8 to 14 days pre-

viously. Landsteiner and Van der Scheer<sup>85</sup> produced anaphylaxis in animals sensitized to azoproteins by injecting the uncombined group, the azo dyes. This work was confirmed by Fierz, Jadassohn, and Stoll<sup>86</sup> by means of Schultz-Dale studies of the uterine strips obtained from guinea pigs sensitized to a diazotized atoxyl preparation.

Landsteiner and Jacobs<sup>76</sup> claimed a relationship between anaphylaxis and skin sensitiveness. Anaphylaxis was produced by injecting picryl-chloride conjugates into animals previously made skin-sensitive to the uncombined chemical. This work was extended by Landsteiner and Chase.<sup>82</sup> By means of intraperitoneal injections with conjugates of picryl-chloride or 2-4 dinitrochlorobenzene, guinea pigs were rendered anaphylactogenic and also achieved a high degree of skin sensitiveness as determined by the surface application of a dilute solution of the uncombined chemical.

While it was the intention of the writer to omit reference to the chemistry of immunological reactions, it was not found possible to do so. The evolution of chemical concepts in immunological phenomenon will be discussed under the next heading.

(4) CHEMICAL HYPERSENSITIVENESS. Obermayer and Pick<sup>87</sup> ascertained that proteins derived from the same species would become heterologous when treated variously by such chemical processes as iodination, nitration, and diazotization. Wolff-Eisner<sup>88</sup> suggested a hypothesis to explain sensitization by protein-free chemical compounds of known composition. He assumed that protein molecules became coupled with the chemical within the body to produce a full antigen and submitted the work of the above authors in corroboration of this postulate. Obermayer and Pick<sup>89</sup> and Landsteiner<sup>90, 91</sup> have placed immunological chemical specificity on a definite basis and, thus, have bridged the hiatus between immunology and chemistry.

In order to present the relation of contact dermatitis to the broad concept of immunochemistry in a concise form, the nature of those chemicals which are known to be common excitants of contact allergy will be discussed first. Then the broader concepts of chemical specificity will be examined.

In the early studies of contact dermatitis, substances of unknown composition were utilized, particularly plant products such as primrose and poison-ivy extracts. But these were later supplanted by chemicals of definite constitution. Bloch,<sup>3</sup> with the help of Karrer, isolated a pure crystalline substance with the formula ( $C_{14}H_{18}O_8$ ) from the primula plant. It possessed the property of producing violent reactions in primrose-sensitive patients. Brown, Milford, and Coca<sup>59</sup> established the oily fraction obtained from short and tall ragweed pollen as the cause of ragweed-pollen dermatitis. It was found, also, that certain related plants contained a common allergenic excitant. Straus<sup>87</sup> obtained reactions with poison sumac in the majority of infants who had been sensitized to poison ivy. The common excitant of these two plants and of the Japanese lacquer tree was found to be urushiol, a mixture of substituted catechols of the average formula  $C_6H_5 \cdot (OH)_2C_{18}H_{27}$  (Mason, *et al.*<sup>92</sup>). It was assumed that these plant substances

were rendered readily accessible to the skin by their miscibility with the natural oils of the skin.

Other allergenic substances, both of defined and less defined composition, have been referred to under a previous heading, "The Sensitizing Substance." Landsteiner and his workers employed chemicals of known composition for the most part. It is important, in correlating the work of these investigators with the topic of contact allergy of the skin, to recognize that they were primarily interested in the specificity of serological reactions and were able to demonstrate that the specificity of antibodies extended beyond proteins to include simple chemical substances. It also was their aim to establish a hypothesis for the immunological phenomena rendered by non-antigenic substances.

In their attempts to sensitize the guinea pig, Landsteiner and Jacobs<sup>17</sup> used various approaches: single or repeated injections; intracutaneous, subcutaneous, or intravenous routes; and direct application to the skin of the chemical in solution or ointment. Tests for sensitivity were made by the intracutaneous method or by direct application of the substance, and the reactions were read in 24 hours. They were recorded in terms of various shades of erythema and grades of infiltration (elevation) of the skin. Vesiculation, present generally in human reactions, was not observed as part of the responses in their animals. The authors stated that sensitization was most readily achieved by direct application of the excitant, and was least successful by the subcutaneous and intravenous routes. By the intracutaneous method, a course of several injections over a period of several weeks was more effective than a single injection. Furthermore, the authors observed that the lesions in animals did not appear to be of the same intensity as those demonstrable in human beings.

In the progression of his studies on specificity of serum reactions along chemical lines, Landsteiner utilized a method of attaching simple chemicals to proteins in order to prepare conjugated antigens containing specifically reacting components of known constitution. At first, acyl groups were introduced into proteins by treatment with anhydrides or chlorides of acids. Subsequently, a more reliable procedure was found to be the joining of proteins with diazonium compounds, designated as azoproteins. The azo-antigen was found to be specific for the azo-component and, to a small extent, for the protein portion of the antigen.

Landsteiner and Jacobs<sup>75</sup> noted a parallelism between the sensitizing capacity and the chemical behavior of certain nitro- and chloro-substitution products of benzene, namely, their lability when treated with alkali. The nitro- and chloro-radicals were loosely bound, so that the parent chemical readily formed substitution compounds with aniline by interacting with the amino group. The authors then postulated that, where sensitization of an organism occurred to simple chemicals, an interaction took place between the latter (hapten or partial antigen) and some constituent in the body, thus giving it antigenic and sensitizing characteristics. The anaphylactogenic property of such conjugated products had been demonstrated by Landsteiner and Van der Scheer.<sup>85</sup> The relationship of skin sensitiveness and

anaphylaxis was claimed by Landsteiner and Jacobs,<sup>78</sup> who produced anaphylaxis through the injection of picryl-chloride conjugates into guinea pigs previously rendered skin-sensitive to the uncombined chemical.

To what extent the above findings can be applied in explanation of the chemical and immunological processes involved in the production of contact dermatitis may be ascertained by a careful analysis of the discussion by Landsteiner himself.<sup>91</sup> He stated that, while conjugation of protein with nitro-substituted benzenes, with acyl chloride, acid anhydrides, and benzyl chloride, substances which are readily conjugated *in vitro*, helped to explain chemical sensitization, it was more difficult to give suitable explanation in the case of such other common allergenic drugs as picric acid, quinine, resorcin, *etc.* He stated, further, that more direct information had accrued from experiments on sensitization with simple chemicals alone, such as primrose extract, orthoform, p-phenylenediamin, mesotan, poison ivy, nitrosodimethylaniline, dinitrochlorobenzene, and others.

Landsteiner likewise contended that, while it seemed logical that results were the same whether the simple incitants or protein conjugates were employed for sensitization, this expectation was not entirely fulfilled. Thus, guinea pigs sensitized intracutaneously with an acyl chloride or picryl chloride reacted with skin inflammation to superficial application of the simple substances and with anaphylactic shock upon intravenous injection of acylated or picrylated protein. Intraperitoneal or cutaneous injection of picrylated stromata, on the other hand, produced anaphylaxis but, at best, very weak skin reactivity to contact. This showed, according to the author, that the two methods were not equivalent and that anaphylaxis and skin allergy to superficial application were two distinct forms of hypersensitiveness.

Further differences, noted by the author, between contact dermatitis and anaphylaxis were that in contact dermatitis there was no transfer by serum, desensitization was not readily accomplished, and, for producing sensitization, treatment of the skin with the simple excitant was far superior to other routes, which in general were of no avail. On the other hand, in common immunization, the resultant effect from treatment of the skin was generally far less pronounced.

In a final interpretation, however, Landsteiner felt that in principle the gap had been bridged as to whether skin sensitization was possible by way of the skin alone or by other routes. Guinea pigs previously treated with killed tubercle bacilli as adjuvants (to aid sensitization) were injected intraperitoneally with conjugates of picryl chloride and red cell stromata, thus achieving a high degree of skin sensitivity, as manifested by surface tests with the excitant (Landsteiner and Chase<sup>92</sup>). From this experiment, the author maintained that skin hypersensitiveness of the contact dermatitis type was engendered by a full antigen and hardly permitted any conclusion other than that this form of allergy was intrinsically related to typical immunization processes.

Considerable discussion has thus been devoted under the last two headings to a presentation of the hypothesis of sensitization by conjugation of simple

chemicals. To what extent this postulate can be applied to the manifestations of contact dermatitis in humans must be left to further investigators in this field. It would appear that the mechanism by which a considerable number of excitants are able to evoke contact allergic responses in man cannot be satisfactorily explained by the above evidence.

Another attribute of chemical compounds in relation to immunological reactions, namely, stereoisomerism, or the spatial structure of chemicals, was likewise studied. Landsteiner and Van der Scheer<sup>93</sup> demonstrated the significance of steric isomerism for the specificity of natural antigens. Employing antigens containing the acyl radicals of the three isomers of tartaric acid, the authors obtained immune sera by which it was possible to differentiate, sharply, optically isomeric compounds attached to the chemicals. Several other experimental and clinical studies pertaining to isomerism in chemical specificity can be mentioned briefly: Dawson and Garbade<sup>94</sup> on drug sensitivity to the alkaloids of quinine; Mitchell<sup>95</sup> on dermatitis to resorcin; Rothman, Orland, and Flesch<sup>96</sup> on procaine dermatitis; and Rostenberg and Kanof<sup>97</sup> on active sensitization with halogen substituted compounds of dinitrochlorobenzene. Another study in point is that of Pauling, Campbell, and Pressman,<sup>98</sup> who demonstrated the greater antigenic effectiveness of para-substituted compounds in comparison to meta- and ortho-compounds in general serological immune reactions. In some of the investigations on contact allergy of the skin, the greater effectiveness of the para-compounds in evoking sensitization was referred to.

In summary, it was demonstrated by the above studies that certain chemicals of known simple constitution were rendered capable of becoming effective sensitizing agents by virtue of their ability to form conjugate compounds with proteins or of the spatial relationship of their component parts (steric isomerism).

### *Summary*

The subject, contact dermatitis, was defined and classified. Factors involved in the mechanism of the development of sensitization were discussed, namely: the sensitizing substance; the role of exposure to the excitant; techniques employed to induce sensitivity; the experimental animal; the flare-up phenomenon; incubation period; sensitization by patch test; susceptibility of subjects; and the state of permeability of the sensitized skin. Experiments which attempted to explain the manner in which sensitization spread from the original site of exposure were described. Finally, the immunology of contact dermatitis and its possible relation to the more recent studies in chemical specificity were presented.

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## ATOPIC ALLERGY: REAGINIC SENSITIVITY

By Matthew Walzer

*Jewish Hospital, Brooklyn, New York*

Atopy, a noncommittal term meaning "strange illness," was originally introduced by Coca and Cooke<sup>1</sup> in 1923 in a theoretical classification of the various forms of hypersensitiveness. It was intended for those forms of human hypersensitiveness, particularly asthma and hay fever, which had been shown to be subject to hereditary influence. The nature of the immunologic mechanism in these conditions was still unknown at the time, but the presence of hypersensitiveness could be detected by skin tests with antigens which produced immediate transient whealing reactions.

In a recent discussion of the origin of the term atopy, Cooke<sup>2</sup> stated: "At that time it was thought that the immediate wheal-reacting allergies and the hereditary group, so-called, were one and the same. Later studies showed that infective or bacterial asthma is likewise and equally subject to the hereditary influence, but does not carry the skin-reacting factor. Today 'atopy' is often used also to designate allergies of the skin-reacting type rather than those with an hereditary factor, and this has caused much misunderstanding."

Coca<sup>3</sup> has always stressed the hereditary implication of the term atopy. Although he and Grove<sup>4</sup> pioneered in investigating the skin-sensitizing antibody, which they named "the atopic reagin," he did not make the positive skin reaction which is mediated by this antibody a prerequisite for application of the term atopy.

Coca now finds his original definition of atopy inadequate in that its designation as "a group of allergic diseases that are subject to common hereditary influence" does not distinguish it from idioblastosis, another category of familial allergic disease which he<sup>5</sup> has more recently reported. Since the mechanism of idioblastosis is not based upon a demonstrable antibody and atopy does commonly manifest the atopic reagin, he offers this feature as a distinguishing characteristic between the two hereditary allergic groups. He therefore modifies his original definition of atopy to read "a group of allergic diseases that are subject to a common hereditary influence and in which the atopic reagins are often demonstrable." Coca has, however, repeatedly pointed out that heredity exercises its influence in various ways on the atopic shock tissue, independently of the reaginic factor, and that it is possible for an atopic shock tissue to be activated by immunologic mechanisms in which reagins play no part. In fact, it is even possible for the atopic shock tissue to be aroused nonspecifically.

It is unfortunate that students in the field of allergy have, for the most part, focused their interest on the skin-sensitizing antibody, while the shock tissue in atopy has received scant attention. Few have grasped the full significance of the fact that, in the absence of a functioning shock tissue, the reagin loses most of its power to mediate allergic symptoms. On the other hand, the shock tissue *can* operate in the absence of demonstrable reagins. Hence, it is important to bear in mind that, while reaginic hyper-

sensitiveness is an outstanding characteristic of atopy, it is by no means a constant feature nor is it the only mechanism involved. The hereditary transmission in atopy applies to tissue predisposition as well as to immunologic tendencies. In other words, atopy and reaginic hypersensitiveness are not synonymous.

The original characterization of atopy as a form of human hypersensitiveness is now obsolete, in view of the demonstration of this mechanism in several species of animals.<sup>6</sup>

There are illnesses other than hay fever and asthma which fall into the category of atopy. Their inclusion is based on their unusually high incidence in patients with asthma or hay fever, either as associated or past illnesses or in their family histories. Most common is the food eczema of infancy and childhood, now commonly known as atopic dermatitis, which is a frequent forerunner or associate of asthma and hay fever and in which the atopic reagin is commonly demonstrated. Perennial allergic rhinitis or vasomotor rhinitis is another common expression of the atopic tendency. Urticaria, angioneurotic edema, and migraine are slightly less frequent but quite common manifestations. In most of these conditions, despite obvious clinical sensitivities, positive skin reactions are usually lacking. It would be difficult, however, to exclude many of these cases from the atopic group solely on this basis. Less typical manifestations of atopy include headache, gastrointestinal disturbances, pruritus, cough, and other symptoms.

Asthma and hay fever each affect approximately from 1.5 to 2 per cent of the population of this country. With the inclusion of the other expressions of atopy, the incidence of the whole group of illnesses is usually estimated at about 7.5 to 10 per cent of the population.

Asthma and hay fever seem to occur with greater frequency among Caucasians than among the other branches of the human family.

Most of the comprehensive studies on the sex distribution of atopic illnesses seem to indicate that there is a slightly higher incidence among males up to the age of puberty and that there is a greater incidence among females thereafter.

The onset of atopic symptoms occurs more frequently in the first decade of life than in any other. Food sensitivities are the earliest to appear, and most frequently they express themselves as eczema or disturbances of the digestive or respiratory tracts. Inhalant sensitivities tend to appear later and to manifest themselves in respiratory illnesses. Most food sensitivities of mild or moderate intensity, starting in infancy or early childhood, tend to disappear spontaneously in the course of a few years. Severe sensitivities to certain food allergens may persist for one or two decades or even longer. Sensitivities to inhalants tend to run a more protracted course than do sensitivities to foods. However, these statements are generalizations. Food and inhalant allergies may appear at all ages in atopic individuals and may run varied and unpredictable courses. Hay fever tends to appear at a somewhat later date than do most of the other reaginic atopic conditions. Its onset is most frequent in the second or third decade. But allergic rhinitis, caused by sensitivities to foods and to inhalants exclusive

of pollens, tends to occur as early as asthma, the onset of which is greatest in the first decade of life.

Atopy has no pathognomic symptomatology or pathology. Conditions simulating atopic illnesses frequently occur in nonatopic individuals. The diagnosis of an atopic illness therefore depends upon the recognition by the clinician of many of the common features of atopy. They include: (1) the more or less classical clinical expressions of the atopic state; (2) the hereditary nature of the illness, expressing its effects, as will be noted presently, in several ways; (3) the pronounced tendency to develop hypersensitiveness, of which the most outstanding and characteristic form is the reaginic type; (4) the prominence of transient localized edema as a finding in most forms of atopic illness; (5) the presence of an excess of eosinophilic leucocytes in the blood and the tendency toward the deposit of these cells at the site of the atopic reaction and in the secretions of the affected mucous membranes; (6) the absence of pathognomic tissue changes on microscopic examination (the edema and tissue eosinophilia being only transitional and completely reversible phases of the reaction and by no means specific for atopic illness); (7) the tendency toward chronicity and recurrence of symptoms; and (8) the relatively benign course of these illnesses, which, in proportion to their chronicity, inflict surprisingly little permanent damage on the affected organs.

Many factors have interfered with the proper performance of statistical studies on the inheritance of atopic illnesses. Despite these handicaps, sufficient evidence has been accumulated to establish, beyond a doubt, the importance of heredity as an etiologic factor in these conditions. The pioneer studies of Cooke, Vander Veer, and Spain,<sup>7, 8</sup> as well as subsequent contributions of other workers, have shown that the influence of heredity expresses itself in several ways.

First, heredity influences the number of offspring to be affected by atopic illnesses. Whereas the incidence of positive family histories among normal individuals has generally been reported to be in the neighbourhood of 7 per cent, it is much higher among atopic patients. The average incidence of positive family histories in a number of series of atopic adults was found to be about 49 per cent. In studies on atopic children, positive family histories occurred in approximately 58 per cent. The number of offspring affected is definitely greater when the hereditary influence is bilateral than when it is unilateral.

Second, heredity exercises its influence upon the age of onset of atopic symptoms. This fact, originally presented in Cooke's<sup>8</sup> studies, has been repeatedly confirmed by subsequent workers. The majority of children with a bilateral atopic inheritance have already manifested their predisposition clinically by the age of ten. During this same period, symptoms appeared in only about half as many children with unilateral family histories, the maximum incidence of onset in this group occurring between the tenth and fifteenth year. In patients with negative family histories, the highest incidence is not reached until about the thirtieth year.

Third, the localization of symptoms and the organs affected are, to a

minor degree, influenced by heredity. Clarke, Donnally, and Coca<sup>9</sup> called attention to the fact that, among atopic patients whose sole complaint was hay fever, the antecedent history for hay fever as a sole expression of the atopic tendency was much higher than that for asthma. Among pure asthmatics, the excess of pure asthma over pure hay fever in the antecedent history was even more pronounced. There are, however, so many exceptions to this general tendency that this expression of hereditary influence does not appear to be so important as those previously mentioned.

Fourth, the atopic individual inherits a predisposition toward reagin production following *casual* and *not unusual* contact with allergens (or atopens, as they are called when used in relation to atopy) which possess no striking antigenic properties. Once initiated, this reaginogenic activity may continue indefinitely, long after antigenic excitation has ceased. The allergens for which reagins are produced are different in parent and offspring, indicating that the *predisposition* to become sensitive, *not* the specific sensitivity, is transmitted. Not all atopic subjects manifest this reagin-forming characteristic. In hay fever, reagins for the offending pollen are almost always demonstrable. In nonseasonal allergic rhinitis, in perennial asthma, and in atopic dermatitis, not more than 60 per cent of patients have reagins. In the remainder, activation of the atopic shock tissues, occurs, directly or indirectly, as a result of nonreaginic allergies, infection, trauma, endocrine dyscrasias, organic pathology, psychosomatic disturbances, physical allergies, chemical irritations, and other processes.

Fifth, there is the inheritance in the atopic individual of what is called, for want of a better term, "predisposed shock tissues," which tend to respond to varied types of excitation with the typical clinical expressions of atopy. Since the tendency to develop atopic symptoms is transmitted much more regularly than the tendency to develop reagins or even to manifest sensitivity, the preeminent importance of the shock tissue as an hereditary factor in the mechanism of atopy is obvious.

The mode of transmission of atopic disease is debatable and promises to remain so for some time. Data on this point will not be trustworthy until all of the possible manifestations of atopy are established, until their actual presence in the patient is recognized, and until two or more generations of many large atopic families are carefully studied.

Hay fever or pollinosis represents an expression of the atopic state which is particularly suitable for study. It is probably the commonest and most readily recognized atopic illness. The offending allergen is a pollen, almost always identifiable by skin tests, the results of which may be corroborated by correlation with the clinical history. In most patients, contact with the offending pollen is unavoidable, and treatment by injections of gradually increasing doses of the offending pollen becomes necessary. For these reasons, the immunologic mechanisms involved in hay fever have been investigated more extensively than those in other atopic illnesses.

Despite the fact that the skin does not participate in the symptomatology of hay fever, the diagnosis in this illness may routinely be made by skin testing, a relatively simple technique in comparison to the more involved

procedure of testing the mucous membranes, which are the seat of the allergic reaction. Skin testing<sup>10</sup> for atopic hypersensitiveness is usually performed either by the scratch or the intracutaneous method or by some modification of these two basic techniques. In the scratch method of testing, introduced by Schloss<sup>11</sup> in 1912, the allergen is gently rubbed into a superficial scratch in the skin. In hay fever, tests are made with pollens, but in other conditions a wide variety of foods, inhalants, or other allergens may be used. In the intracutaneous technique introduced by Cooke,<sup>7</sup> minute amounts of sterile extracts of allergens are injected into the skin. With both techniques, positive reactions consist of wheals, usually irregular in outline, with a tendency to pseudopod formation. The wheal is usually surrounded by an erythema and accompanied by itching. Positive reactions start to develop within a few minutes, reach their height in about ten to fifteen minutes, and disappear in about an hour. For diagnostic purposes, either or both of the techniques are commonly employed, depending upon the preference of the clinician and the nature of the problem. In the hands of the novice, the scratch technique is the safer method of testing. The intracutaneous technique is more effective in diagnosis, but requires greater experience and care, because antigens are actually introduced into the body with this procedure. For research purposes the intracutaneous test has proved the more adaptable and effective technique.

Both methods of testing are based upon the same immunologic mechanism, first experimentally demonstrated by Prausnitz and Küstner<sup>12</sup> in 1921. Küstner was clinically sensitive to fish and manifested a marked positive reaction on skin test with this allergen. The introduction of a small amount of Küstner's serum into Prausnitz's skin resulted in a localized sensitization to fish at the injected site. This was proved experimentally by an intracutaneous test with the fish allergen the following day. This skin-sensitizing property was shown by Coca and Grove<sup>4</sup> to be more or less typical of sera obtained from atopic patients who showed positive skin reactions to the common atopens. Because the sensitizing substance contained in these sera manifested properties which differed decidedly from those manifested by the sensitizing antibody in anaphylaxis, the term "atopic reagin" was suggested for it.

The tissue-sensitizing property represents virtually the only one by which the atopic reagin may be detected and identified. Hence, almost all studies of this antibody involve, at some point, direct skin testing of the patient or the use of the Prausnitz-Küstner technique or some modification thereof.

It has been experimentally demonstrated that sensitization of tissue cells starts within a few minutes after the introduction of the reagin-bearing serum into the skin and is completed within a few hours. This induced local sensitivity starts to decline gradually after a week or so, but, with high-titered sera, evidences of sensitivity may persist for as long as eight or ten weeks after sensitization.

The intensity of sensitization induced at a site depends, to a considerable degree, on the reagin concentration of the serum. In untreated patients with hay fever, Levine and Coca<sup>13</sup> found a definite proportional relationship



between the degree of the patient's cutaneous sensitivity and the reaginic titer of his blood. This is not regularly true in cases of asthma and other atopic illnesses.<sup>14</sup>

Not all individuals accept passive sensitization to the same degree. Generally speaking, atopic subjects are less receptive to passive local sensitization than are nonatopics.<sup>15</sup> The factors responsible for these variations in receptivity are unknown. Mucous membranes may also be passively locally sensitized by intramucosal injections of reaginic sera.<sup>16</sup>

For the testing of passively sensitized skin sites, about 0.02 ml. of allergenic extract is injected intracutaneously, as superficially as possible. The trauma produced by the sensitizing injection of serum temporarily diminishes the responsiveness of the skin site. For this reason, the test is best performed after an interval of three or four days.<sup>3a</sup> A positive reaction consists of a wheal or an erythema, usually both, which are not obtained with a control test performed on an unsensitized site. This opportunity to compare similar tests on sensitized and unsensitized sites represents the greatest advantage of passive transfer testing. No similar control is afforded in direct testing on the patient.

A marked positive passive transfer reaction affects the sensitized cutaneous site in several ways.<sup>17</sup> It impairs the whealing response of the tissue to any type of subsequent test for from one to four weeks. In addition, the positive reaction injures *all* the reagins remaining at the site, including those not related to the antigen. Hence, one marked reaction at a sensitized site materially reduces the value of all subsequent testing at that site. The stronger the reaction, the less reliable are the results of subsequent retests of the site. The failure to realize this simple fact has resulted in reports of bizarre findings in experiments in which the passive transfer technique or its modifications were employed.

The atopic reagin possesses properties which differ, in many respects, from those of the anaphylactic or precipitin antibody. The latter, while playing an important role in animal hypersensitiveness, is of limited significance in human allergy. A comparison<sup>3b</sup> of the two types of antibodies reveals the following points of differentiation.

(1) The atopic reagin is decidedly more susceptible to heat than the precipitin. By heating serum at 56°C for an hour, it is possible to eliminate its reagin activity. Such treatment does not seriously affect precipitin antibodies.

(2) The atopic reagin totally lacks the power of sensitizing guinea-pig smooth muscle, a cardinal characteristic of the anaphylactic antibody. The latter, on the other hand, manifests little of the human skin-sensitizing property so characteristic of the atopic reagin.

(3) Mixtures of reaginic serum and related antigen yield no visible precipitates, such as are produced in the proper mixtures of anaphylactic antibodies with their respective antigens.

(4) Fixation of complement by mixtures of reaginic serum and antigen occurs irregularly and only with selected sera.<sup>18</sup> It is transient in comparison to the readily demonstrable, more permanent fixation which occurs with

mixtures of precipitin and antigen. Moreover, the zone of antigen dilution in which this phenomenon occurs is different for the two types of antibodies. The reaginic reaction is operative in zones of much higher antigen dilution than the precipitin reaction.

(5) Compared to the strong affinity existing between the anaphylactic antibody and its related antigen, the attachment between unanchored reagent and its antigen is an extremely loose one and is reversible to an unusual degree.

A twenty-four-hour mixture of reagent and related antigen in the proper dilutions, when introduced into the skin, produces, within twenty minutes, a specific positive reaction, indicating that there has been an attachment of reagent to tissue cells even in the presence of an excess of antigen. The sensitized cells are then acted upon by the antigen, resulting in wheal formation and complete or partial loss of site sensitivity. So slight is the loss of antigenic power in the test tube mixture and so striking is the positive reaction resulting from its intracutaneous introduction that some workers have denied the occurrence of any *in vitro* reaction and have maintained that all neutralization of reagent occurs *after* the introduction of the mixture into the skin. This seems unlikely, since Bowman has found that twenty-four-hour mixtures of reagent and antigen produce smaller immediate reactions in the normal skin than do those mixtures prepared just before injection. The fact that fixation of complement has been demonstrated with reaginic sera also supports the belief that some antigen-reagent reaction takes place *in vitro*, even though the combination is weak and is, to a large degree, reversible.

Reagent production is not readily induced experimentally with the food and inhalant antigens, which are the common excitants of atopic symptoms. Even in atopic subjects, who would be naturally predisposed to form reagents, Brunner<sup>19</sup> failed, with one questionable exception, to stimulate their production by repeated subcutaneous injection of large amounts of egg white, rabbit epithelium, and orris root, all common clinical atopogens.

With *Ascaris lumbricoides* antigen, which possesses unusual antigenic properties, Fülleborn,<sup>20</sup> Brunner,<sup>19</sup> and others showed that reagent formation could be readily induced in nonatopics as well as in atopics by relatively few skin-test doses of the extract.

In an attempt to study some of the factors which might influence reagent production, a series of studies on sensitization to *Ascaris* in humans was undertaken by Davidson, Kailin, and co-workers.<sup>21</sup> Five groups of subjects, among whom none showed positive skin reactions to *Ascaris*, were studied successively. Skin-test doses of *Ascaris* were administered at weekly intervals until an immediate positive skin reaction developed to the test, at which time reagents could be demonstrated in the blood by passive transfer test. About 19 per cent of the subjects developed immediate positive reactions to *Ascaris* at the time of their third weekly skin-test dose, which was fourteen days after their first contact with the antigen. About 54 per cent were sensitive by the twenty-eighth day and about 92 per cent by the seventy-seventh day. The rate of reagent formation to was *Ascaris* definitely

faster in the Negro than in the white race. There was suggestive evidence that males were more readily sensitized to than *Ascaris* females. Chronic tubercular infection did not appear to be a factor influencing the rate of sensitization. Among adults, age was not a factor. The rate of sensitization in any group could not be correlated with the natural incidence of positive reactors resulting from parasitic infestation in that population group. The artificially induced reagins to *Ascaris* persisted for more than six months after sensitization in more than half the cases tested, with a tendency to longer duration in the Negroes.

It is impossible to state, at this time, whether the race and possibly the sex factors which seemed to influence artificial sensitization with *Ascaris* allergen will be found to apply to reagin formation with the common excitants of atopic illnesses. Reagins stimulated by nonparasitic antigens may appear in normals as well as in atopics following parenteral injection of immune animal sera, insulin, liver extract, and other products of animal origin, but their production is irregular and unpredictable.

It is apparent, therefore, that atopic reagins are not pathognomic of atopic illnesses. There is, nevertheless, the striking tendency exhibited by the atopic individual to become sensitive to common atopens possessing no unusual antigenic properties in animals and to stay sensitive, frequently for years after contact with the excitant has been discontinued. It is this predilection to reagin formation which facilitates the diagnosis of the atopic hypersensitive state by means of skin testing.

Unfortunately, the presence of the skin-sensitizing antibody and the positive skin reaction which it mediates are not an absolute index of the patient's clinical sensitivity to the particular atopen in question. While a positive skin reaction to an atopen usually may be correlated with an active sensitivity to it, the reaction frequently bears no obvious relationship to symptomatology. The reaction may be a forerunner of clinical sensitivity or the residual evidence of a previous sensitivity. This lack of correlation may be attributable in part to the patient's failure to contact the atopen in its active state, or through natural channels, or in sufficient concentration to be effective. In addition, there are inactive or anergic states of the shock tissue which render it insusceptible to excitation by the immunologic mechanism. Such anergic states may occur spontaneously or follow specific treatment. They are also frequently produced nonspecifically by various forms of therapy or by fever, infections, pregnancy, and severe tissue damage, such as fractures or surgical procedures. Hence, positive reactions, even though they are specific, need careful interpretation.

No less important is the evaluation of negative reactions. Frequently in tests with foods and quite regularly with drugs, the skin reaction is negative, although clinical sensitivity to these substances is pronounced. It is obvious that there is more to the art of specific diagnosis in atopic illness than the mechanical performance of the skin test.

In addition to its importance in the direct testing of patients, the skin-sensitizing power of the atopic reagin has been employed for diagnostic and experimental purposes in many other ways. From the practical point of

view, it is being employed for the indirect testing<sup>20</sup> of atopic patients in whom direct testing is inconvenient or impracticable. By sensitizing many skin sites on the arms of a normal subject with small amounts of the patient's sterile serum, it is possible to test these areas, after an interval of a few days, with those allergens with which the patient would ordinarily be tested.

Another use of passive local sensitization has been in studies of antigenic absorption.<sup>22</sup> A cutaneous site, sensitized with a reaginic serum of high titre, will react violently, within five minutes to an hour, following the oral administration of the related antigen, and the entrance of minute traces thereof into the circulation. With this technique, it has been possible to demonstrate the presence of traces of unaltered antigen in the circulation, following its application to practically all mucous membranes, serous surfaces, and even to the skin.

Passive sensitization of human mucous membranes has been employed in studies of reaginic reactions in these tissues. Among the most interesting of these was the allergic reaction of the passively sensitized ileum and colon.<sup>16</sup>

From the investigative point of view, attempts at passive sensitization of the common laboratory animals with human reaginic sera were uniformly disappointing until Caulfeild and Straus almost simultaneously reported success with the Rhesus monkey. The skin and mucous membranes of this animal are readily sensitized with human sera of high titre, and this has afforded an opportunity for many unusual types of investigation. Of singular interest has been the study of allergic reactions in the abdominal organs of the monkey.<sup>23</sup>

Man accepts passive cutaneous sensitization with the sera of some animals. However, the intracutaneous injection of these heterologous sera is usually followed by rather marked inflammatory reactions which render the skin sites useless for purposes of testing. Hence, relatively little has been learned from the experimental use of reagin-bearing animal sera in man.

In anaphylactic shock in the guinea pig, rabbit, and dog, the seat of the reaction is constant and characteristic for each animal species. The atopic reaction in humans involves no such characteristic shock organ. In animal anaphylaxis, the role of smooth muscle as the shock tissue is readily demonstrable with the Schultz-Dale technique, employing the excised smooth muscle strip of the uterus or intestines. No similar evidence implicating human smooth muscle has yet been presented. Tuft<sup>24</sup> obtained a human uterine muscle segment from a patient who had been sensitized and showed a positive skin reaction to horse serum. Atopic reagents to horse serum were present in her circulation. Tests of this muscle strip with the Schultz-Dale technique yielded no specific contractions to horse serum.

Attempts to sensitize the smooth muscle of laboratory animals with human reaginic sera have consistently failed. Even in the Rhesus monkey, the best animal receptor of sensitization with human reagents, Albert<sup>25</sup> could find no fixation of reagents in the smooth muscle of the intestinal strip, although large amounts of potent reaginic sera were employed for sensitization. There was, likewise, no fixation of the anaphylactic antibody by the smooth muscle in this animal.

Despite these consistently negative findings, there has been considerable reluctance, in some quarters, to relinquish the hypothesis that smooth muscle is the seat of the immunologic reaction in atopic illnesses. In support of this concept, the bronchial spasm in asthma and the spasm of the bowel in gastrointestinal allergy are offered as evidence. But, here again the experimental proof that the immunologic reaction actually takes place in the smooth muscle is lacking. Not even the smooth muscles in the blood vessels of sensitive mucous membrane contract during the atopic reaction. In slit lamp studies of positive ophthalmic reactions induced with pollens in hay fever patients, Feldman and Sherman<sup>26</sup> observed only vascular dilatation during all phases of the specific response.

Further evidence against smooth muscle as the shock tissue in gastrointestinal allergy is found in the roentgenographic studies by Fries and Zizmor.<sup>27</sup> After a barium meal containing a specific allergenic offender, the characteristic response of the allergic stomach consisted of a marked loss of tonicity and a pronounced decrease in peristalsis, resulting in a long delay in emptying of the organ. In the allergic intestines, spasm was the more typical finding, but dilatation was by no means an uncommon occurrence. The fact that the smooth muscle relaxes regularly in the allergic reaction of the human stomach, and occasionally in that of the bowel, eliminates it as the shock tissue in these phenomena, since Schultz-Dale studies reveal that smooth muscle strips of sensitive animals invariably contract in response to antigenic contact. Any muscular contractions which occur in bronchial asthma or gastrointestinal allergy are, therefore, secondary responses to the allergic reaction which occurs in the neighboring mucous membranes or other tissues.

The exact site of the immunologic reaction in atopic hypersensitiveness is still a matter of conjecture. While the particular type of cells affected in humans may be constant, the organs in which these reactions occur vary widely, much more so than in laboratory animals. Even in the same individual there is frequently a tendency for alternation in the involvement of shock organs. The eczema of infancy and childhood commonly gives way to some other atopic manifestation, such as asthma or hay fever, in later life. Occasionally, two atopic symptoms, such as asthma and eczema, may alternate several times in the life of the same individual. The factors responsible for these variations in susceptibility of the shock tissues are still obscure.

There is also little known concerning the factors which are responsible for the particular pattern of sensitivities which each patient develops. There is evidence to suggest that the development of sensitivity to an atopen is not accidental. The absorption into the circulation of unaltered food and inhalant allergens has been shown to be a normal physiologic phenomenon occurring throughout life in atopic and nonatopic subjects alike.<sup>28</sup> With variable amounts of allergens constantly reaching the circulation by way of the digestive and respiratory tracts, an explanation for the establishment of sensitivity on the basis of accidental excessive absorption of a particular antigen becomes untenable.

The tendency, commonly manifested by atopic patients, to develop multiple sensitivities to certain types of antigens, with the allergenic pattern varying for each individual, speaks for a selectivity in sensitization, probably the result of some predisposition. From the studies of Harten and Bowman,<sup>28</sup> one is led to the conclusion that, among patients with clinical manifestations of hay fever, there is a pronounced tendency to develop reagins, though to a variable degree, to the strong pollen excitants to which they are exposed. Such a predisposition to sensitization to allergens of a particular type is commonly observed in patients sensitive to animal danders, fish, cereals, legumes, meats, *etc.*

The importance of antigenic contact as a prerequisite to reagin formation was brought out in the early studies of Grove,<sup>34</sup> who reported complete absence of sensitivity to ragweed, the most important American pollen excitant, in hay fever patients in Germany, where that plant does not grow. Similarly, Algeroba pollen, the most important offender in Hawaii, gave uniformly negative reactions in hay fever patient in New York, where the plant does not exist.

One must regard with suspicion reports of reagin formation without previous contact with an antigen. In such cases, it is likely that contact has occurred, but in a manner not recognized or remembered by the patient. There is the additional possibility of a previous contact with an antigen closely related to the one in question and possessing decisive antigenic components in common with it.

Passive sensitization of the fetus, *in utero*, a phenomenon readily demonstrable in guinea pigs, does not occur in humans. Bell and Eriksson's<sup>29</sup> report that maternal reagins, unlike other antibodies, do not pass through the placenta into the fetal circulation has been repeatedly confirmed. No one has demonstrated the presence of atopic reagins of any sort in the newborn. Even when reaginic formation and cutaneous sensitivity were actively induced with *Ascaris* antigen in pregnant women, Zohn<sup>30</sup> could find no trace of atopic reagins in the cord blood at birth.

Proof is still lacking for the hypothesis that active intra-uterine sensitization occurs in humans, as it does in guinea pigs, as a result of contamination of the fetal blood by antigen from the maternal circulation. In such a case, it would be necessary to demonstrate the presence of atopic reagins at birth or during the first two or three months of life. In the latter instance, it would be necessary to establish a complete absence of contact with antigen after birth. Since Donnally<sup>31</sup> and also Brunner<sup>32</sup> demonstrated the excretion of unaltered food allergens into the mother's milk, it would be necessary to exclude this source of antigenic contact between birth and the appearance of sensitization.

The role of histamine in various allergic reactions has already been discussed and needs receive only brief consideration here. The exhaustive experiments of Lewis<sup>33</sup> and his co-workers revealed a marked resemblance between the characteristics of the immediate cutaneous whealing reactions induced by pricking the skin with histamine and with a specific allergenic excitant. This led Lewis to conclude that the action of the antigen upon

the specifically sensitive tissue cells resulted in the liberation of a substance which exhibited a marked similarity to histamine. This histamine-like substance, which Lewis termed H-substance, was said to damage the minute skin vessels, causing an increase in their permeability. The Lewis theory probably suffers from oversimplification, in that it fails to account for many experimental phenomena noted by other investigators in this field. In our own experience,<sup>34</sup> the histamine theory fails to account for the dissimilar conditions of responsiveness which prevail at the sites of histamine and specific wheals produced on the same atopic individual. For many days after the initial excitation, the site of a histamine wheal shows a diminished responsiveness to restimulation with histamine. The specific wheal, on the contrary, leaves its site with an increased responsiveness to restimulation, not only with the specific excitant but also with histamine. There is also the curious fact that identical histamine skin tests injected along the arm produce wheals of gradually increasing size as one proceeds downward, while specific tests with atopens behave in the opposite manner.<sup>35</sup> There is the additional finding, determined by electrophoretic studies,<sup>36</sup> that successful specific treatment of hay fever patients with pollens does not alter the threshold of their cutaneous responses to histamine. It is difficult to reconcile these findings with the histamine theory proposed by Lewis.

Attempts to approach the treatment of atopic illnesses by immunization against histamine in one form or another have, on the whole, been disappointing. Histamine injections have proved of limited value in cases with reaginic sensitivity. Immunization with histamine-azo-protein, employed in the hope of stimulating antibody formation against the hapten, histamine, such as Fell<sup>37</sup> claimed to have produced in rabbits, has failed to yield beneficial results. The use of histaminase, aimed at the enzymatic destruction of histamine *in vivo*, is theoretically unsound and has proved to be valueless clinically.

The antigenic substances or atopens which are the specific excitants of atopic reactions are diverse in nature. The identity and chemical nature of the excitant is still uncertain in many of the most important allergens, including such prevalent offenders as pollens and house dust. For this reason, little progress has been made in the methods of standardization of materials used for the testing and treatment of atopic illnesses. Many of the important atopens are proteins of a complex nature and of large molecular size. In the case of pollens, however, there is evidence to indicate that they are of comparatively small molecular size and that they manifest the properties of large-sized complex polypeptides.<sup>38</sup> Despite the fact that pollen is a relatively poor anaphylactogen, it is a potent and extremely important atopen.

In atopic sensitivity to drugs and chemicals, reagins are almost uniformly absent. Several notable exceptions to this rule have recently been reported. Feinberg<sup>39</sup> found evidences of reaginic sensitivity to drugs among workers in a factory where sulphonechloramides were prepared. The two most important offenders were chloramine T and halozone, simple chemical substances of low molecular weight (211). These drugs produced asthma and

allergic rhinitis in fourteen workers who had handled them for from six months to ten years before they developed symptoms. Most of the patients had positive personal or family histories of atopy and showed positive skin reactions to other atopens. In four of the six patients tested, marked passive transfers to chloramine T in high dilution were obtained. A case of reaginic sensitivity to a sulfonamide was recently reported by William Sherman, and reagins to phthalic anhydride were found in a chemist by Kern. In all of these cases, the patients showed immediate positive reactions to direct tests with the chemicals.

With rare exceptions, bacteria and bacterial products do not yield immediate positive skin reactions of a reaginic nature. Delayed skin reactions are likely to follow skin tests with bacterial allergens.

The treatment of atopic hypersensitiveness may be approached in several ways. Probably the simplest and most effective method is to eliminate contact, if possible, with the offending allergens. In most food allergies, this not only results in rapid clearing of symptoms, but, if continued long enough, is usually followed by a loss of sensitivity. The latter outcome is less easily achieved with inhalant sensitivity.

When exposure to the excitant can not be easily avoided, as is the case with most pollen-sensitive cases, treatment with the specific offender is indicated. When the pollinating season is already on, small daily intracutaneous doses of the pollen may alleviate symptoms. Preferable, however, is the preseasonal form of treatment involving subcutaneous administration at four- to seven-day intervals of gradually increasing doses of pollen, varied according to the patient's tolerance. The protective effect of dosage wears off rapidly, so that preseasonal treatment must be repeated each year, unless the patient continues treatment perennially, receiving his maximal dosage at four-week intervals indefinitely. The latter practice seems to offer the most satisfactory results. Hay fever treatment by the oral administration of pollen is theoretically unsound and disappointing in practice. All forms of "rush" treatment, which attempt to hasten or force dosage more rapidly than the patient's tolerance will permit, are hazardous and are generally discouraged.

The fact that specific treatment produces only partial and temporary benefit is further evidence that it does not desensitize the atopically sensitive human in the same sense that the anaphylactic guinea pig may be desensitized by proper doses of antigen. The treated patient who has obtained a satisfactory result tends to show no loss of reagins but may even exhibit a slight increase in the reagin titre of his serum. This finding, first reported by Levine and Coca<sup>13</sup> and repeatedly corroborated since, turned the quest for information on the mechanism of specific treatment in other directions.

Cooke<sup>40</sup> thought he had found the answer when he reported, in 1935, the detection of a so-called "blocking antibody" which appeared in the serum of hay fever patients as a result of treatment with pollen. Mixtures of pollen and post-treatment serum, when injected into normal skin, gave little or no immediate reactions, while mixtures made with pollen and ante-treatment



serum produced strong immediate positive reactions. These findings and the results obtained on retests of the injected sites led Cooke to believe that treatment had produced a new antibody which blocked the antigen-reagin reaction in the tissues. Subsequent studies<sup>41</sup> revealed that these antibodies were formed in nonsensitive nonatopic humans following a series of pollen injections. As he continued to study the problem, Cooke's<sup>42</sup> enthusiasm waned, until he eventually doubted that the blocking antibody was responsible for the clinical protection afforded by specific treatment.

Loveless,<sup>43</sup> who had collaborated with Cooke in his earlier studies, took a different view of the matter. In independent studies, she demonstrated that the antibody in the post-treatment serum is thermostable, in contrast to the reagin, which is quite susceptible to heat. She showed, furthermore, that thermostable antibody, in competing with reagin for the same antigen, was the more successful of the two in combining with and neutralizing the antigen. This accounted for the differences which Cooke had observed in the behavior of ante-treatment and post-treatment sera and which he had attributed to a blocking action of the antibody.

Loveless<sup>44</sup> confirmed the findings of Cooke, Rackemann, and others that there was no direct correlation between the absolute titer of the thermostable antibody resulting from treatment and the clinical improvement of the patient. She denied the importance of this objection, however, on the ground that she believed the increase in titer, necessary to produce improvement, varied with each patient.

Alexander and Johnston recently devised an original capillary tube precipitation technique involving tests with human sera, antigen, and immune rabbit serum, which provided another method of investigating this problem. Their first studies<sup>45</sup> seemed to indicate that relief of hay fever tended to correspond with the elevations in titer of the thermostable antibody. In their latest report on a large series of cases, however, these workers were not inclined to reaffirm their first impressions.

It seems likely that an alteration in the response of the shock tissue is the most important factor responsible for the clinical improvement induced by the treatment of hay fever. This is most effectively obtained by specific treatment in one form or another but, as previously noted, may be temporarily achieved even through nonspecific means. Just why or how the shock tissue in atopy becomes inactivated following treatment remains as much of a mystery as the reason for the spontaneous recovery from atopic illnesses even though the reaginic mechanism persists. These are among the major problems which today challenge the investigator in the field of atopic illnesses.

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## ALLERGY OF INFECTION: RELATION TO IMMUNITY

By Beatrice Carrier Seegal

*Department of Bacteriology, College of Physicians and Surgeons,  
Columbia University, New York*

The accelerated vaccinia reaction or "disposition to sudden cuticular inflammation" was noted by Jenner<sup>1</sup> some 100 years before von Pirquet<sup>2</sup> reinvestigated the reaction as an example of an allergic condition. The term allergy had been introduced by von Pirquet<sup>3</sup> only one year previously. Thus a vivid description of hypersensitivity to an infectious agent preceded the present knowledge of allergic reactions by a full century.

Zinsser<sup>4</sup> has defined bacterial allergy as "a condition in which the body is sensitized to a bacterial antigen." This definition makes the problem seem disarmingly simple, although actually it is quite the contrary. In addition to producing all the known types of hypersensitive reactions, previously described under anaphylaxis, the products of infectious disease agents stimulate certain reactions which are generally peculiar to this type of allergen. Some, perhaps much, of the histopathology in chronic infection may be the result of allergic inflammation.

Two factors at least contribute to the varied manifestations of the allergy of infection. The first is related to the intricate structure of pathogenic microorganisms and their many biological activities which enable them to invade and excite inflammatory reaction. The second is a result of the host's response to infection. Experiments will be cited which indicate that the type of tissue in which the organism lodges and the reaction it evokes may influence the character of the allergic manifestations.

*Anaphylactic Hypersensitivity Induced by Bacteria and Fractions of Bacteria.* Studies made with whole microorganisms or with their fractions or products have shown that bacteria or their products may induce anaphylaxis in animals in a manner similar to cells or proteins of non-bacterial origin. Friedberger and Mita<sup>5</sup> described anaphylaxis induced by whole bacteria. Guinea pigs injected subcutaneously with suspensions of heat-killed *Vibrio melchnikovii* were fatally shocked 2 to 4 weeks later by intravenous injections of the same material. Recent observations with whole dead bacteria are those of Boldt, Tanner, Rosebury, and Kabat at Camp Detrick.<sup>6</sup> They found that subcutaneous injections of killed suspensions of *Brucella suis* or *Bacillus anthracis* sensitized guinea pigs so that symptoms of anaphylactic shock could be induced either by intravenous injection of the antigen or by exposing the animals to inhalation of clouds of the bacteria.

The first virus purified, crystalline tobacco mosaic virus, can sensitize the guinea pig so that anaphylactic shock follows the intravenous injection of the virus.<sup>7</sup> It has not been possible to demonstrate smooth muscle sensitivity by the Schultz-Dale technique in such animals.<sup>8</sup>

Whole bacteria may induce the Arthus type of sensitivity. Baker, Thomas, and Penick<sup>9</sup> sensitized rabbits by intracutaneous injection of living hemolytic streptococci. Subsequent intrapericardial injection of a heat-

killed culture of the same organism has resulted in extensive pericarditis and myocarditis similar to the Arthus reaction induced with soluble protein.

The phenomenon of passive anaphylaxis may be demonstrated with antiserum to whole bacteria or to bacterial fractions. Avery and Tillett<sup>10</sup> have passively sensitized guinea pigs with rabbit antipneumococcus serum and elicited fatal shock in these animals with the type specific carbohydrate. Lancefield<sup>11</sup> has studied the antigenic nature of fractions of the hemolytic streptococcus by this technique, using guinea pigs passively sensitized with rabbit antistreptococcus serums.

Another series of experiments has been designed to test the capacity of fractions of bacteria to produce allergic reactions in previously sensitized animals. For example, Enders<sup>12</sup> has utilized the "partial antigen" of the tubercle bacillus to induce anaphylactic shock in actively and passively sensitized guinea pigs. Mackenzie<sup>13</sup> has investigated anaphylactic sensitivity to pneumococcus filtrate. Guinea pigs received intraperitoneal injections of killed or living broth cultures of virulent pneumococci which produced active immunity. Anaphylactic shock might follow intravenous injection of a filtrate of the pneumococcus, and a positive Schultz-Dale uterine contraction was obtained when pneumococcus protein was added to the bath. Cutaneous allergic reactions were not obtained.

Fractions of bacteria may induce the Arthus reaction. Francis and Tillett<sup>14</sup> have reported that the specific capsular carbohydrates of pneumococcus Types I, II, or III, when injected intracutaneously in rabbits which had been immunized actively or passively to the whole organism, induced a delayed inflammatory response, the Arthus reaction. Zinsser<sup>4</sup> elicited what may be interpreted as a similar reaction by injecting pneumococcus autolysate into the joints of sensitized guinea pigs. Benacerraf and Kabat,<sup>15</sup> in their quantitative studies of the passive Arthus reaction in the guinea pig, have utilized horse antipneumococcus Type I serum to sensitize passively to the purified Type I polysaccharide.

It is possible both to sensitize and to shock with fractions of bacteria. For example, Sugg, Lurline, and Neill<sup>16</sup> have used diphtheria toxin and toxoid as anaphylactogens. Corper<sup>17</sup> has similarly demonstrated the anaphylactogenic activity of a protein from the tubercle bacillus.

It is interesting to observe that passive sensitivity with heterologous sera is not uniformly successful. For example, by the usual technique horse antipneumococcus serum will not passively sensitize the guinea pig so that anaphylactic shock results, following intravenous injection of the specific carbohydrate. Nevertheless, a local wheal and erythema reaction<sup>18</sup> or an Arthus reaction<sup>16</sup> can be demonstrated if the carbohydrate is injected intracutaneously. Francis and Tillett<sup>14</sup> have shown that, when a rabbit receives antipneumococcus horse serum and is subsequently tested by intracutaneous injection of the specific carbohydrate, a delayed type of reaction, characterized by edema, erythema, and sometimes purpura, follows.

*Allergic Reactions Considered Characteristic of Infection.* Allergic reactions following infection with the tubercle bacillus have been extensively investigated and constitute a classical example of bacterial allergy. The

intracutaneous injection of tuberculin in tuberculous animals produces the well-known tuberculin reaction, which, in time of appearance and duration, is similar to the Arthus reaction, the local inflammatory reaction which follows the subcutaneous injection of non-bacterial antigens in sensitive animals. The tuberculin reaction, however, though indurated, usually shows less gross edema and more hyperemia than the Arthus reaction. According to Rich<sup>19</sup> these two reactions differ in histopathology. The most unequivocal difference between them lies in the fact that the serum of the tuberculous animal cannot transfer the tuberculin reaction passively to a normal animal, whereas the passive transfer of the Arthus reaction with the serum of a sensitive animal is readily accomplished.

If tuberculin is injected in large amounts in tuberculous animals, focal and constitutional reactions occur and tuberculin shock and death may follow within a day or two. Like the tuberculin skin reaction, tuberculin shock is not transferable to a normal animal with the serum of a sensitive animal.

Tissue damage resulting from exposure of infected animals to tuberculin may be conveniently demonstrated by instillation of this allergen in the conjunctival sac. This has been practiced as a useful diagnostic test in cattle. Conjunctivitis is noticeable within a few hours, reaches its maximum intensity in 16-48 hours, and is severe, requiring almost a week to heal completely. Another type of tissue damage which may be readily demonstrated is inhibition of spermatogenesis following the intratesticular injection of tuberculin in sensitive animals.

*In vitro*, in tissue culture, the cells from tuberculin-sensitive guinea pigs and rabbits are prevented from migrating by the presence of an amount of tuberculin which is innocuous to the cells from a normal animal.<sup>20</sup> This hypersensitivity to the specific antigen is not found in tissue culture cells from animals sensitive to non-bacterial antigens.<sup>21</sup> Kirchheimer and Weiser<sup>22</sup> and Heilman and Feldman<sup>23</sup> have studied the correlation between cutaneous reactivity to tuberculin and sensitivity of tissue culture cells to the same allergen. The former workers found that the cells of splenic explants from guinea pigs desensitized to the intradermal tuberculin test were resistant to the cytotoxic action of tuberculin, when compared with cells from tuberculous, non-desensitized animals. Heilman and Feldman, using tissues from rabbits which had developed negative tuberculin reactions, due to overwhelming infection with the *Mycobacterium tuberculosis* or to intercurrent infections, found no corresponding resistance to tuberculin in their tissue cultures. Fremont-Smith and Favour<sup>24</sup> have extended *in vitro* studies of the cytotoxic effect of tuberculin to observations on bloods from human patients and have contrasted the behavior of these cells with those from mice and guinea pigs. They report an interesting difference in the behavior of lymphocytes and neutrophils. The former, if from tuberculous man, mouse, or guinea pig, are lysed by tuberculin. The latter cells are lysed only if from man or guinea pig.

The results of the tissue-culture studies suggest that the antibody which reacts with tuberculin must be closely bound to the cells. This finds con-

firmation in the observation of Chase<sup>25, 26</sup> that the cells of tuberculin-sensitive guinea pigs are capable, when injected into normal animals, of passively sensitizing this host to tuberculin. Peritoneal exudates, spleens, lymph nodes, or blood from animals rendered hypersensitive by the injection of dead organisms provided the cells. Kirchheimer and Weiser<sup>26A</sup> have employed cells of guinea pigs sensitized by the injection of living cultures of the BCG strain of *Mycobacterium tuberculosis* and have obtained the same results.

In experimental studies of allergic phenomena resulting from inoculation with the streptococcus and pneumococcus, Andrewes, Derick, and Swift<sup>27</sup> and Julianelle and Morris<sup>28</sup> have reported an interesting spontaneous skin reaction called a "secondary" reaction. Rabbits were injected intradermally with certain strains of streptococci or pneumococci, either living or dead. After the primary injection of the bacteria, a small edematous and erythematous lesion developed, which healed within a week. When the spontaneous secondary reaction appeared, it came 8 to 10 days after the initial injection. It was characterized by the development of areas of edema and erythema at the old sites of inoculation, which sometimes became even larger than the original ones. Furthermore, ophthalmic sensitivity and a tuberculin-like shock could be demonstrated following the appropriate injection of the specific bacteria about two weeks after the beginning of the experiment.<sup>29</sup> Neither of these latter two reactions were transferable to a normal animal with the serum of a sensitive animal.

Another example of cell hypersensitivity, demonstrable *in vitro*, is furnished by splenic explants from guinea pigs suffering from a chronic Group C hemolytic streptococcus infection. Moen<sup>30</sup> exposed such tissue-culture preparations to filtrates of the infecting agent and demonstrated inhibition of migration and damage to the explanted hypersensitive cells.

*Influence of the Host's Cellular Response on the Development of Bacterial Allergy.* During infection there are a variety of cellular reactions on the part of the host to the invading bacteria. There is evidence from the work of Dienes and Schoenheit<sup>31</sup> that this cellular reaction may of itself influence the demonstrable allergic phenomena. They injected a fraction of a milligram of egg white into tuberculous lesions in rabbits or guinea pigs. Subsequent skin testing of these animals with the egg white resulted in a *tuberculin* type of skin reaction. The implication of the observation would seem to be that the cellular response to the infection by the tubercle bacillus influenced the development of allergy to the egg white so that it now behaved as a bacterial allergen. The same result was accomplished when the egg white was injected in a focus of infection produced by the vaccinia virus.

Recently the experiments of Dienes and Schoenheit have been extended by Raffel, Arnaud, Dukes, and Huang.<sup>32</sup> The latter group had made the observation that the injection in guinea pigs of a wax derived from the tubercle bacillus, together with proteins from this organism, established a tuberculin type of sensitivity to the proteins. When wax of the tubercle bacillus, together with egg albumin, was injected into guinea pigs and later

the animals were skin-tested with the egg albumin, a tuberculin-like reaction developed. Also, a delayed inflammatory response in the cornea followed the injection of the albumin in this site. These reactions were not demonstrable in animals sensitized with egg albumin alone. Bone-marrow cultures showed killing and lysis of cells by egg albumin when the explants were obtained from guinea pigs sensitized with the combination of wax and albumin.

The possible influence which focal lesions may exert on the character of bacterial allergy was illustrated in the experiments of Derick and Swift.<sup>29</sup> They tested for the ophthalmic reaction and the lethal tuberculin-like shock in rabbits which had received their initial inoculation of streptococci by a variety of routes. The intravenous route failed to promote either of these types of allergic reactions. On the other hand, primary inoculation into other areas, such as the knee, muscle, or peritoneal or pleural cavities, was followed, in a proportion of animals, by the development of such sensitivity. The authors conclude that it is probable, since this type of bacterial allergy seems to accompany the production of focal lesions, "that in these foci are produced the substances or conditions which lead to this type of bacterial allergy."

*Summary: The Varieties of Bacterial Allergy.* The experiments which have been described illustrate the following points: Infectious agents and their products may serve to produce anaphylactic shock, the Arthus reaction, and the Schultz-Dale reaction. In addition, infection with microorganisms, or injection of suitably chosen portions of these agents, may yield an allergic state which can be characterized by the induction of a tuberculin-like skin reaction, a delayed ophthalmic reaction, a tuberculin-like focal reaction, and, finally, delayed tuberculin-like shock. In contrast to the anaphylactic reactions, these latter cannot be transferred by the serum of the sensitive animal to normal animals. Cells of a tuberculous animal, however, are capable of passively sensitizing a normal animal. Tissue cultures of cells from infected animals may be inhibited in growth by extracts of the specific infectious agent. When a simple foreign protein, egg albumin, is injected into a focus of infection or together with a wax from the tubercle bacillus, it acquires many of the characteristics of a bacterial allergen.

*Naturally Occurring Bacterial Allergy in Man.* When the natural history of bacterial allergy in man is considered, it is desirable first to observe what evidence there is of an allergic response to bacteria in presumably normal, healthy individuals. Mackenzie and Hanger<sup>30</sup> have studied the development of sensitivity to filtrates of hemolytic and of viridans streptococci. Infants up to the age of ten months do not react to those filtrates. Thereafter, the percentage of positive reactions increases with age. It did not prove possible to correlate such reactions with known infections. Stevens and Jordani<sup>31</sup> have skin-tested asthmatics with nucleoproteins of *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Neisseria catarrhalis*, *Haemophilus influenzae*, and *Streptococcus viridans*. Both the immediate wheal and erythema type of reaction and the delayed tuberculin-like reaction were ob-

tained. In six instances only did both types of reaction follow a single inoculation. Usually a patient, on repeated testing, reacted in the same manner to a given extract with either the immediate or the delayed type of reaction. A person might, however, give an immediate reaction to one extract and synchronously a delayed reaction to another extract. The intensity of the reactions fluctuated. The incidence of a tuberculin-like skin reaction to the nucleoproteins of the hemolytic streptococcus have been observed in infancy, childhood, and adult life by a number of investigators.<sup>55</sup> The reaction, rarely positive in infancy, becomes increasingly frequent with age. These observations illustrate the occurrence of allergic responses to organisms which may be carried in the upper respiratory tract.

Positive dermal reactions to products of the infectious agent have been demonstrated during or following many diseases caused by viruses, fungi, or parasitic helminths as well as by bacteria.<sup>26</sup> Such reactions may or may not be associated with immunity. For example, as a case of pneumonia approaches convalescence, a tuberculin-like skin reaction to the pneumococcus nucleoprotein is demonstrable and also a wheal and erythema type of reaction to the type specific soluble carbohydrate.<sup>36</sup> This latter reaction is associated with the development of immunity. Some allergic reactions presumably contribute to the pathology of infection. As stated by Zinsser,<sup>4</sup> "A body allergic to a given bacterial antigen is vulnerable to a degree that may lead to a serious pathological change." It is important to determine if these undesirable inflammatory reactions can be avoided and immunity preserved. This requires an examination of the relation of allergy to immunity.

*Allergy and Immunity.* The observation of Koch that a previously infected guinea pig, allergic to tuberculin, is capable of localizing and destroying reinjected tubercle bacilli has contributed largely to the point of view that allergy is necessary to immunity in tuberculosis. Rich<sup>19</sup> has challenged the dependence of immunity upon allergy in this disease on the basis of a series of experiments in which Rothschild, Friedenwald, and Bernstein<sup>37</sup> desensitized tuberculous animals to tuberculin and found their immunity unimpaired. Other studies by Rich and his associates<sup>38, 19</sup> present evidence that immunity to experimental *Treponema pallidum*, *Pneumococcus* (Type I), and *Pasteurella aviseptica* infections is also independent of allergy. Rich therefore concludes that allergy and immunity are unrelated. Allergic inflammation, however, is the result of antigen-antibody reaction in the tissues. Immunity also depends, in part, upon the action of antibodies upon the invading microorganisms. In short, both phenomena represent *in vivo* tests for the presence of antibody. It is difficult, on theoretical grounds, to believe that they are separable.

It would seem that, in designing an experiment to test the relation of allergy to immunity, the first prerequisite is to determine which antigen of the given microorganism is associated with invasiveness. Antibody to this antigen is the factor which determines immunity. This information is available for only a limited number of microorganisms, notably for such encapsulated organisms as the pneumococci, *H. influenzae*, and the Fried-



länder's bacillus, for the *Str. hemolyticus* group A, and probably for certain enteric organisms.

Little work has been done which is directly designed to correlate immunity with allergy to the specific antigenic fraction of a bacterium responsible for infection. Protection against infection in mice with the Group A hemolytic streptococcus is achieved by the use of antiserum containing antibodies to the type specific M protein. The studies of Bailly<sup>39</sup> concerning hemolytic streptococcus (Type 30) infection in rabbits were designed to examine the question of the relation of allergy to immunity in streptococcus disease. Rabbits which had circulating precipitins and positive tuberculin-like skin reactions to both the type specific M protein and the group specific C carbohydrate were desensitized by the intravenous injection of either the M protein or the C carbohydrate. Precipitins and skin tests to the antigen used for desensitization became negative. Those rabbits desensitized with C carbohydrate, but with circulating antibodies and positive skin tests to M protein, proved to be immune to infection with the type 30 streptococcus. Those animals desensitized with M protein, although still having positive skin tests and antibodies to the C substance, lost their immunity to the type 30 streptococcus and died, when they were challenged with this organism. In this case, had only the allergic reaction to the C carbohydrate been considered, immunity would have appeared to exist in the absence of allergy. When the allergy to the vital M protein was studied, however, immunity was lost when allergy was lost.

It is not yet known which of the numerous constituents of the tubercle bacillus is associated with the capacity to infect. The tubercle bacillus possesses at least five proteins, two carbohydrates, and also phospholipids.<sup>40</sup> Tuberculin (PPD) is one of the five proteins. There is reason to believe that tuberculin can be excluded as a factor in virulence, since the capacity to produce tuberculin is not limited to virulent organisms.<sup>41</sup> If, then, tuberculin does not determine invasiveness, antibodies to tuberculin cannot protect, nor can their absence deprive the host of immunity. Hence, allergy to tuberculin is a mark of immunity only in so far as it is correlated with the presence of an immunizing antibody. Desensitization to tuberculin should be expected to leave immunity to infection intact. It is even possible that desensitization with tuberculin might have a beneficial effect on this disease, provided the desensitizing injection was not responsible for focal or constitutional reactions.

In summary, it would seem that the problem of the relation of allergy to immunity is one that can be properly studied only in those infections where the particular antigenic fraction of the organism responsible for virulence is known. Furthermore, the experimental data presented here indicate that more basic knowledge concerning the factors which lead to bacterial invasion and host response must be at hand before many of the problems relating to the allergies of infection are solved. Indeed, only recently tools of sufficient precision have been available. The newer methods of immunochemistry make possible the antigenic analysis of bacteria, the association of one fraction with virulence, the quantitative estimation of antibodies,

and the analysis of types of reaction between antigens and antibodies. As these methods are applied to this field, the problems of the allergies of infection and their relation to immunity will be clarified. At the moment the field is almost entirely unploughed, due, in part, to the diversion of man power to the meadows of antibiotics. Yet it has more than academic interest, for chronic diseases in which allergy may play such a significant role are sometimes those as yet uncontrolled by the chemotherapeutic agents. Furthermore, information gained concerning allergy in infection must add also to the understanding of other allergic diseases. This constitutes one of the real frontiers of medicine and a challenge to the newest methods of immunochemistry.

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# IMMUNOLOGIC CHANGES BROUGHT ABOUT BY FUNGI AND FUNGOUS PRODUCTS

By Marion B. Sulzberger

*Professor and Chairman of Department of Dermatology and Syphilology, Skin and Cancer Unit and Post Graduate Medical School of New York University—Bellevue Medical Center.*

From a very early date, research on the immunologic reactions produced by fungi has served as a sort of pioneer system of experimentation for the investigation of infections in general. Interest in experimentation with fungi lies not solely in the importance of fungous diseases themselves but also, as Bloch pointed out as early as 1908, in the fact that studies of fungous infections are such excellent simple means for investigating the immunologic happenings which occur in infections in general and that, in particular, there is such a close analogy, and in many respects identity, between the results of immunologic studies with fungi and those with the tubercle bacilli.

Paradoxically enough, the recognition of pathogenic fungi can be said to have been the very beginning of the bacteriologic era. Indeed, Schoenlein's recognition of the genus *Achorion* as the cause of favus represents the first sure identification of a microorganism as the causal factor of a human disease. Further, one may fix the beginning of the immunology of fungous diseases as far back as 1902, when Plato and Neisser produced and employed trichophytin extracts analogous to the crude tuberculin of Koch. Very early, Gruby, and much later Sabouraud, two French dermatologists, studied the clinical manifestations of fungous infections of the skin, hair, and nails. They engaged in the clinical description and the cultural and botanical classification of the various types of fungi found in man and concluded that different diseases were caused by different fungi, not by the same fungus. Sabouraud showed that each species of fungus had a tendency to produce a particular form of disease.

After this great advance, however, the subject was dead for many years. It appeared that most of the known fungi pathogenic to mammals had been classified and that nothing very startling or new remained to be described. This stagnation lasted until 1907 or 1908, when Bloch introduced the animal experiments into the studies of fungi. This inaugurated a long series of studies by many men. Then came a great list of workers, topped by J. Jadassohn, one of the originators, including Jessner, Truffi, Martenstein, Biberstein, W. Jadassohn, Saeves, Kogoj, and many others.

Animal experiments with dermatophytic fungi were soon found to have substantial advantages over experiments with many other forms of pathogenic microorganisms. For example, the animals were not killed by the disease, nor was absolutely exact dosage required. The disease was practically confined to the skin and its appendages, so that everything that happened could be observed directly and at desirable intervals, histopathologically, microscopically, immunologically, and in many other ways. Then there were no special precautions to be taken in the laboratory to pre-

vent human infections or cross-infections among the experimental animals. Above all, there was usually a uniform consistency and predictability in the course, development, and regression of the disease. To my knowledge, there is no other experimental disease where there is such fine regularity and so little variation in time intervals as there is in the skin and hair infections of the guinea pig with *Achorion quicqueanum*.

#### *Experimental Results and Inferences After Infections and Reinfections*

With such experimental fungous infections and reinfections of the skin of laboratory animals with *Achorion quicqueanum*, many fundamental immunobiologic principles of infection have been demonstrated with the utmost clarity and at a very early date. Many of the immunobiologic findings preceded those in tuberculous and other infections. Indeed, many immunologically significant facts have been demonstrated exclusively in experimental fungous diseases. Thus, the wealth of immunologic lore is far greater in relation to fungous infections than to any other infectious diseases affecting man. The following are selections from among the most important findings.

*First Infection.* On first infection there is a regular incubation period which lasts about 10–14 days. At the end of this period, the phenomena noted include the following: (1) clinical and histopathologic inflammatory changes appear at the inoculation site; (2) as a rule, the trichophytin skin test (intradermal injection and 24–48 hour reading) becomes positive, roughly coincidentally with the first appearance of clinical manifestations; (3) as a rule, the more inflammatory the lesions, the greater the trichophytin sensitivity of the skin; (4) as a rule, the more inflammatory the lesions, the greater the tendency to local healing and the less easy it is to demonstrate fungi by direct microscopic or by culture methods.

When one performs trichophytin skin tests at various intervals after healing of the local lesions, one finds that the trichophytin hypersensitivity acquired as the result of the infection is usually long-lasting, often persisting for the lifetime of the animal.

*Reinfection.* If one takes a guinea pig which has recovered from a previous infection and reinfests the animal at another skin site with the same fungus, one finds that there is occasionally, perhaps rarely, a complete immunity and failure to produce disease. Usually, however, an infection of modified form and course takes place at the reinfection site, the lesion appearing within a few hours, instead of after an incubation period of many days. Moreover, the healing sets in much earlier after reinfection than after the first infection. The recovery is generally complete in from 10 to 12 days, instead of about 21 to 28 days. The obvious reason for this accelerated course is that the incubation period (*i.e.*, the period needed for the development of immunologic changes which lead to trichophytin hypersensitivity) is no longer required in the *reinoculated* animal, which has retained a persistent trichophytin hypersensitivity as a result of its previous infection.

*Inferences.* It is from findings of this kind that the following inferences may be drawn:

(1) The development of hypersensitivity to the fungous allergens contained in "trichophytin" is intimately connected with the appearance of the inflammatory disease.

(2) The degree of trichophytin hypersensitivity of the tissues is intimately connected with the severity of the manifestations, the time intervals required for the disease to appear and to run its course, the tendency to healing, and the number of fungi present and demonstrable in the lesions.

In translating these inferences to human disease or to infections in general, one may arrive at the following postulates:

(1) The diseases produced by these groups of fungi (hyphomycetes-dermatomycetes) are probably *not* caused by toxins or poisons elaborated by the microorganisms, but are most likely caused by fungous *allergens*. The diseases are thus, from their inception, immunologic or allergic sensitizations, in this respect, just like anaphylaxis or hay fever or contact-type eczematous dermatitis from plants, dyes, or other simple chemicals. Moreover, as shown by Bloch, Schaaf, and Labouchère in 1924, one of the most important allergenic principles in these fungi is a polysaccharide and no at protein.

(2) The fungous diseases of this type are, thus, analogous to numerous important diseases, such as tuberculosis, leprosy, and syphilis, in that, in these latter also, the microorganisms are not known to produce any toxins or intrinsically damaging substances, but do produce allergens or immunologically active substances which sensitize the tissues and which then give rise to the local allergic reactions which constitute the principal manifestations of the disease.

(3) On the basis of these principles, presumably, one can divide microorganisms and the diseases they produce into several categories:

(a) Allergenic microorganisms and the principally allergic diseases, such as fungous infections, tuberculosis, leprosy, syphilis, and many others.

(b) Toxin- or poison-producing microorganisms and the diseases they produce, such as diphtheria, tetanus, *etc.*

(c) Microorganisms which have combinations of allergenic and toxic action, *e.g.*, streptococci in scarlet fever, certain staphylococci, *etc.*

(d) Microorganisms producing disease by still other mechanisms (*e.g.*, competition for and withdrawal by the microorganism of substances vitally needed by the tissues of the host; synergistic or summation effects of microorganisms plus other agents; and mechanical or chemical destruction, or obstruction, embolization, coagulation, lysis, *etc.*).

While I should like to enlarge upon the many clinical and theoretical significances of the facts and inferences just sketched, I shall have to confine myself to just a few points of practical importance.

In contrast to what is done in achieving antitoxic immunity in the toxin-forming infections, if the ideas expressed are correct, the allergic infectious diseases should, in theory, best be prevented and treated by desensitization or hyposensitization measures, in analogy to the manner in which anti-anaphylaxis is produced in animals or hay-fever patients are desensitized or hyposensitized with the specific pollen allergens. Indeed, as Wise and

Sulzberger showed in 1932, in many cases of fungous diseases, the skin's sensitivity to trichophytin can be substantially, if only temporarily, reduced by repeated intracutaneous injections of trichophytin. Also, in some cases, there was a concomitant improvement of the fungous disease. However, we stated that the method, at present, was of negligible therapeutic or practical value because of the many unpredictabilities. Included among these was the uncertainty as to which patients, instead of a lowering, would develop a great increase in trichophytin sensitivity and a concomitant worsening and/or spread of their allergic fungous disease. Moreover, even when the level of the skin's sensitivity to trichophytin could be reduced by the repeated injections, this reduction was usually transitory, there being a strong tendency for each skin to return to its previous "natural" level of sensitivity. Here again, the many resemblances to the results of tuberculin desensitization in the treatment of tuberculosis will be obvious.

Even a cursory contemplation of the findings and their implications should show that it is fruitless to argue about whether the hypersensitivity to allergens of microorganisms (trichophytin or tuberculin hypersensitivity, *etc.*) is protective or harmful. If the author is correct, then the hypersensitivity is, first of all, the *sine qua non* of the disease and thus, in that sense, unequivocally and obviously harmful. However, connected with, and perhaps resulting from, this very hypersensitivity and the resulting tissue reactions, many forms of beneficial and protective tissue reactions can occur, for example: the more rapid local destruction and throwing-off of the microorganisms; the inflammatory demarcation of the affected sites; the retardation or prevention of dissemination and spread of microorganisms and their products; and the inflammatory mobilization and *taxis* to the affected site of many other means of protection (*e.g.*, hyperemia, lysins, agglutinins and other protective antibodies, leucocytes and lymphocytes, *etc.*).

Thus, the hypersensitivity is: (1) very harmful locally; and (2) may be one of the great beneficial and protective factors to the host as a whole. It is, however, in most instances, neither one nor the other exclusively.

The findings, that the higher the degree of trichophytin sensitivity which a fungus produces, the greater the local inflammation, the more difficult it is to demonstrate the microorganisms in the tissues, and the greater the tendency to rapid course and spontaneous healing, all help to explain why certain fungi are more inclined than others to cause little or only superficial inflammation but are highly contagious from man to man, producing seemingly mild diseases which are, paradoxically, often the most difficult to cure.

The fungi of this group I have termed "anthropophilic", because of their predilection for man, contrasting them with the "zoophilic" fungi, *i.e.*, those which prefer to infect lower animals, but which, when they do cause sporadic cases in man, produce greater trichophytin hypersensitivity, greater and deeper inflammation, and a more distinct tendency to spontaneous cure.

These observations and classifications are of practical significance just

now in regard to the difficulties of controlling the present epidemic of scalp infections of children with the anthropophilic, poorly sensitizing *Microsporon audouinii*, in contrast to the sporadic cases of more inflammatory, more readily curable scalp infections with the more highly allergenic *Microsporon lanosum* or *Microsporon canis*.

It is, moreover, noteworthy that *Trichophyton purpureum*, another fungus with a low potential for producing sensitizations with 24–48 hour inflammatory-type reactions to trichophytin (but which, as shown by George M. Lewis and co-workers, produces mainly immediate, wheal-type sensitizations), is also the cause of some of the most stubborn and therapy-resistant forms of superficial fungous disease of the skin.

I should like, at this point, to go into the many other immunologic facts and findings in which experiments with fungi and fungous allergens have done the pathfinding. For example, I should like to present, with the detail they merit, the studies of J. Jadassohn and his school on the immunologic basis of local and hematogenous first infections, reinfections and superinfections; those of M. Jessner on the very early immunity in experimental sporotrichosis; and those of Stephan Epstein and collaborators, who showed that a zone of local specific immunity forms at the center of the "ringworm" patch on human skin and that such an immune zone also forms at and beyond the border of the enlarging patch. Thus, in Epstein's classic studies, specific but local immunologic changes were proven to occur and were shown to account for the configuration of certain characteristic skin lesions, as well as for the noteworthy and fortunate circumstance that a local infection usually does not spread to cover the entire body surface but stops enlarging when it reaches a certain extent, mainly because it builds up a circumferential immunity which blocks its own further extension. I should have liked also to discuss the pathogenesis and immunology of the secondary eruptions known as trichophytids, as demonstrated by J. Jadassohn, Bloch, W. Jadassohn, and co-workers. Most particularly, I regret that space does not permit me to give proper exposition to the basic and significant findings of W. Jadassohn and his collaborators on the relationships of fungous hypersensitivity to anaphylaxis and on the use of the Schultz-Dale experiment and the Prausnitz-Kuestner experiment for the demonstration of the characteristic but complex and composite mosaic of the allergenic principles developed by each different species, strain, and family of fungi.

Due to lack of space, however, I must refer the reader to the very adequate literature on these subjects, appended herewith in the bibliographic list.

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# FAMILIAL NONREAGINIC FOOD ALLERGY

By Milo G. Meyer

*Gardner Medical Group, Michigan City, Indiana*

Familial nonreaginic food allergy, or "idioblapsis," owes its name to Dr. Arthur F. Coca. It is a highly controversial subject. If the assumptions which, for the most part, Dr. Coca and I believe to be true become established facts, the mystery as to the etiological factors of previously unexplained illnesses will be removed. If, however, we should be found mistaken, those of us who are successfully using the knowledge of this allergy as a therapeutic aid are running the risk of professional condemnation and ridicule. Further, we could only attribute the results obtained to the effect of psychological suggestion on unstable and neurotic patients. This paper, then, is written not in defense of the existence of such an allergy but, rather, out of my own experience, in an attempt to explain further the rationale of the conclusion that there is an extraneous factor which not only precipitates a disordered mechanism of bodily function but, also, renders the organism susceptible to the ravages of certain infectious agents.

Of necessity, constant reference must be made to Dr. Coca's<sup>1</sup> book on this subject. Having had the opportunity of testing his radical ideas in a certain group of patients typical of those seen by the average internist, I believe very serious consideration of his theories is warranted. Thus, this paper will be divided into the following sections: (1) theory; (2) practical application; (3) results obtained. From this division, it is hoped to prove the existence of idioblapsis as an entity.

## *Theory*

"Idioblapsis" is derived from blapsis, a "spoiler," and idio, which conveys the meaning of individual peculiarity. Familial nonreaginic food allergy, as pointed out by Coca, adequately expresses the thought, were it not for the fact that other excitants besides foods also conform to the fundamental requirements of its definition. TABLE 1 illustrates the important aspects in which this group of allergies differs from atopy, allergy of infection, serum disease, and contact dermatitis.

We contend that the symptoms and illnesses shown in TABLE 2 are generally derived from food allergy. It is obvious to all that there are the complaints which comprise the vast majority of illnesses seen in the average physician's office and for which, to date, only symptomatic therapy is being given. Some of these have, in Dr. Coca's experience and in my own, responded so brilliantly to exclusion therapy that we believe there is little doubt as to allergic etiology.

This phase of allergy is not well worked out. Most allergists depend on the cutaneous reaction as a means of solving the individual's problems. The other method is to subject the patient to the unwieldy and discomforting process of a long series of elimination diets. The latter falls short of the expected goal because food sensitivity is a very individual problem,

and, as we shall show later, diets do not take into consideration that any one food whatsoever may be the causative agent in the patient's illness. The cutaneous test, according to figures which Dr. Coca and I have checked, fail in their diagnostic ability in this phase of allergy more than 75 per cent. This will be shown in a later table.

The incidence of atopy among the food-allergic patients studied by Coca seems to be fixed at about 5.7 per cent in young people. This is shown in

TABLE 1  
CHARACTERISTICS OF IDIOBLAPSIS

1. The hereditary influence controlling its occurrence is independent of the atopic inheritance.
2. Allergic antibodies (reagins) are not demonstrable.
3. Many of the symptoms are not represented in the atopic group.
4. The allergic reaction practically always causes acceleration of the pulse.

TABLE 2

SYMPTOMS PROVISIONALLY RECOGNIZED AS FOOD-ALLERGIC IN 54 PATIENTS. ALL SYMPTOMS WERE ACCOMPANIED BY TACHYCARDIA AND ALL DISAPPEARED AFTER ELIMINATION OF THE FOODS THAT CAUSED TACHYCARDIA

<i>Symptoms</i>	<i>Number of patients affected</i>	<i>Symptoms</i>	<i>Number of patients affected</i>
Headache, migraine (7)	38	Irritability.....	3
Headache, severe (11)		Chest pain.....	3
Headache, mod. (20)		Abdominal pain.....	3
Physical tiredness.....	36	Angioneurotic edema....	3
Nervousness.....	23	Gastrointestinal bleeding.....	2
Indigestion, including gas, vomiting, nausea.....	21	Pain in gall-bladder.....	2
Dizziness.....	20	Conjunctivitis.....	2
Constipation.....	16	Gastric pain.....	2
Neuralgia.....	15	Angina pectoris.....	2
Canker sores.....	13	Diarrhea.....	2
Chronic rhinitis.....	13	Colitis.....	1
Heartburn.....	11	Anorexia*.....	1
Urticaria.....	9	Chronic cough.....	1
Epileptiform seizures.....	7	Dysmenorrhea.....	1
Overweight.....	6	Frequent epistaxis.....	1
Psychic depression.....	3	Nervous and emotional instability.....	1
Extra systoles.....	6	Chronic bronchitis.....	1

\* Possibly due to deficiency of vitamin B.

TABLE 3. Independently of this, I have a small series of sixty cases in which atopy appears in about 18 per cent. This is shown in TABLE 4.

The rather high incidence, I believe, may have been due to extremely careful questioning, which may have made the patient desirous of attempting to support positive answers to my questions. These figures are obtained by history, not by skin testing. It would seem, then, if one is correct in the assumption that we can explain the patient's symptoms on the basis of allergy, to date at least, that the sensitization of the cardiovascular appara-

tus is the only accurate way of arriving at a satisfactory opinion as to the allergens involved. TABLE 5, taken from Coca, indicates the results of cutaneous tests. (Note that only in MMD, CB, and JJV, *all asthmatics*, positive tests appear; in the remainder, direct intra-cutaneous tests and tests in substitute are negative. This percentage is much smaller than the 30 per cent which I originally indicated.) In an attempt to confirm this in a small way, ten patients of my own are shown, in whom the pulse dietary tests as well as intracutaneous and scratch tests were used (TABLE 6).

Although several other observers<sup>2</sup> have recognized the existence of a curative effect by the elimination of certain foods, the percentage of success, when a diagnostic dependence is placed on cutaneous tests, will of necessity

TABLE 3

SHOWING THE OCCURRENCE OF ATOPY AMONG 191 FOOD-ALLERGIC PERSONS AND 69 PERSONS FREE OF FOOD ALLERGY

Familial nonreaginic food allergy.....	191	(74.5%)	Atopy....	11	(5.7%)
No food-allergy.....	69	(25.5%)	Atopy....	4	(5.7%)
Total.....	260	(100.00%)	Atopy....	15	(5.7%)*

Patients who have been under the dietary treatment for nonreaginic food allergy are not included in this survey. The individuals making up the group were taken at random among residents in a suburban town (Oradell, New Jersey) and among nurses in the nearby Hackensack hospital.

\* No doubt the smallness of this percentile incidence of atopy is due to the fact that the group is composed largely of children and young adults.

TABLE 4

THE OCCURRENCE OF ATOPY AMONG 50 FOOD-ALLERGIC PERSONS AND 10 PERSONS FREE OF FOOD ALLERGY, ALL OVER AGE 30

Familial Nonreaginic Food Allergy..	50	Atopy.....	9	(18%)
No Food Allergy.....	10	Atopy..	1	(10%)

be small. Since the pulse response is the diagnostic criterion concerned, we must consider its mechanism. This is now only speculative. It is apparently well agreed that there is an independence of the shock organs or areas; that is, that each organ or system retains an independence of reaction to the insulting substance. This would strongly suggest that the reacting substance is cell-bound or sessile, but does not explain why, when excitants are avoided for a comparatively long time, the reaction to their next insult is lacking. The acceleration of the pulse, according to Coca, is specific in every case. He feels this so strongly that he makes no exception. The specificity is apparently almost 100 per cent. I am sure, however, that I have seen two patients who have proven food allergy and yet I have failed to demonstrate an increased post-prandial heart rate. FIGURES 1 and 2 indicate this observation. These are the *only* two cases so far encountered and they are not sufficient to disprove Coca's theory, since both cases were responsible for their own pulse rates.

In an attempt to build a platform on which to explain the sensitization of the cardiovascular system, one may turn to the histamine theory. We

then assume that the shock tissue, when insulted, liberates a histamine-like substance which increases the heart rate, either because of blood vessel caliber alteration following the sympathetic nervous system stimulation

TABLE 5

RESULTS OF CUTANEOUS TESTS IN 32 SUBJECTS OF FAMILIAL NONREAGINIC FOOD ALLERGY  
FIGURES INDICATE THE NUMBER OF DIFFERENT FOODS USED IN THE TESTS; ALL OF  
THOSE FOODS HAD CAUSED TACHYCARDIA IN THE RESPECTIVE PERSON

<i>Patient</i>	<i>Direct intracutaneous tests</i>	<i>Indirect tests in a substitute</i>
E. F. C.....		Negative (3)
A. F. C.....		Negative (8)
M. M. D. (asthma and hay- fever).....	{ Negative (4) Positive (1)	
C. T.....	Negative (5)	Negative (4)
J. G.....	Negative (2)	
A. R.....	Negative (1)	
L. R.....	Negative (3)	
S. H.....		Negative (3)
W. W. F.....		Negative (2)
M. F.....		Negative (3)
M. A.....		Negative (6)
P. W.....		Negative (4)
E. B.....	Negative (2)	
C. B. (asthma).....	{ Negative (2) Positive (2)	
R. M.....	Negative (5)	
J. J. V. (asthma).....	{ Negative (9) Positive (3)	
J. K.....	Negative (11)	
J. F.....	Negative (8)	
W. S. C.....	Negative (3)	
J. V.....	Negative (13)	
M. N.....	Negative (4)	
R. F.....	Negative (7)	
N. vW.....	Negative (9)	
L. H. B.....	Negative (21)	
Mrs. E. B.....	Negative (6)	
A. S.....	Negative (15)	
M. S.....	Negative (6)	
W. G.....	{ Negative (2) Neg.-sl. (1) not passively Slight (1) transferable	
M. P. age 11 (grandmother, G. B. asthmatic).....	{ Negative (6) Positive (1 and dust)	
G. B. (asthma).....	Negative (3)	
E. K.....	Negative (2)	
G. H.....	Negative (16)	

In some patients tests were not made with some of the known allergenic foods. Negative tests with nonallergenic foods are not included.

or by a direct effect on the cardiac accelerator or inhibitory center. This may also explain the loss of pulse acceleration after repeated ingestion of the offending food. Several investigators<sup>3</sup> have shown that the response to histamine itself is quantitatively lessened after either repeated injections at the same local site or by the injection of one large dose in saline. This in-

TABLE 6  
COMPARISON OF PULSE-CRITERION AND SKIN TEST IN TEN ALLERGIC PATIENTS

Patient	Age	Symptom complex	Allergens causing tachycardia	Allergens by skin tests
H. B. ....	62	Rhinitis	Potatoes, beef, corn	Pollens, only has seasonal hay-fever
L. N. ....	38	Migraine	Tomato, egg, oat, coffee	None (histamine 4 plus)
B. G. * ....	16	Acne	None	Chocolate, egg
V. H. ....	34	Asthma	Beef, egg, orange and wheat	Egg, corn, rye, milk, dust pollens
R. J. ....	24	Pruritus	Egg	None
A. K. ....	52	Hypertension	Egg, coffee, banana, cereals	None
M. M. ....	50	Hypertension	Potato	None
C. L. ....	38	Hypertension	Dairy Products, nuts	None
F. M. ....	62	Hypertension		Veg. oil, feathers, dander, dust, wool
M. R. ....	28	Pruritus generalized Arthritis	Pork, corn	
		Ménière's syndrome	Pears, peaches, apples	None

Note that a case of acne failed to show pulse acceleration, although skin tests to chocolate and egg were positive. The acne has *not* cleared with the elimination of chocolate and egg. In one asthmatic, tachycardia and whealing to egg are shown. In no other case is there similarity.

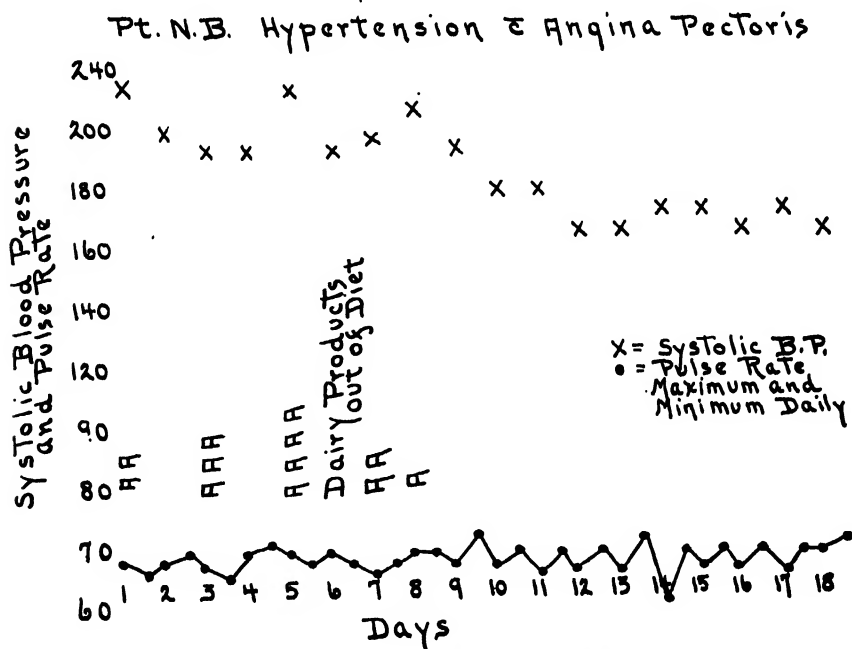


FIGURE 1. The hypertension in NB is apparently relieved with elimination of dairy products; yet, according to the pulse chart which she kept, there is no variability in rate before and after elimination.

creased tolerance persists for as long as twelve hours, probably much longer. This reaction must be nonspecific as far as the sensitizing agent which liberates a histamine-like substance is concerned. That fact was established by Farmer<sup>4</sup> in 1922. I have attempted a correlation of pulse rate before and after histamine injections with certain allergens remaining in the diet. The result in three of my patients is shown in FIGURE 3.

The exact mechanism involved is, therefore, not completely rationalized and the premises given are only suggested. That the autonomic nervous system is involved in this increased pulse rate is very strongly suggested by the stability of the pulse rate after the severance of the sympathetic chain

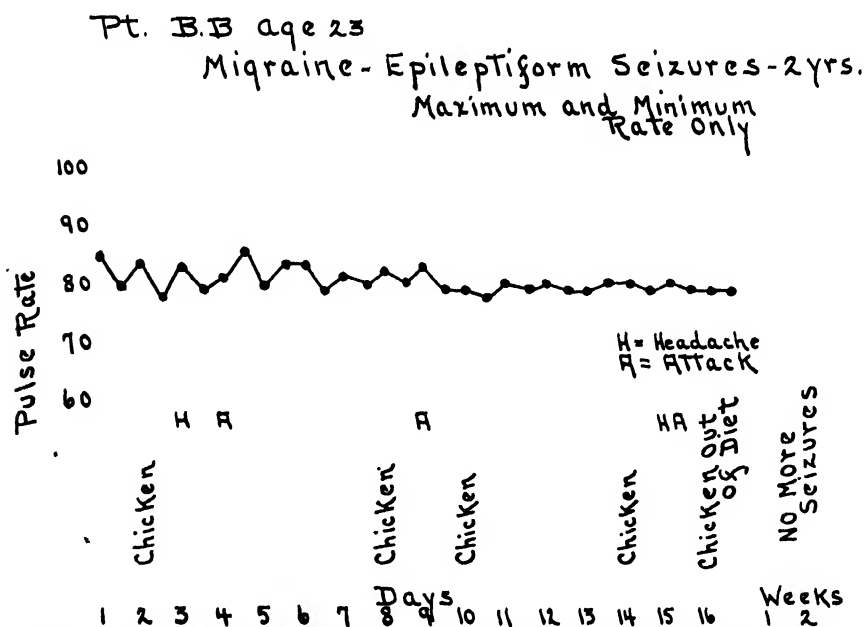
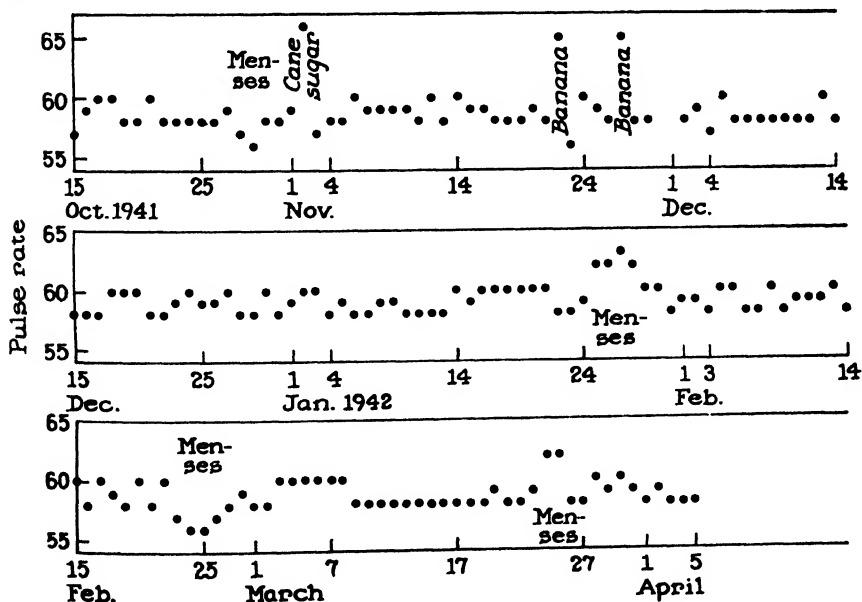
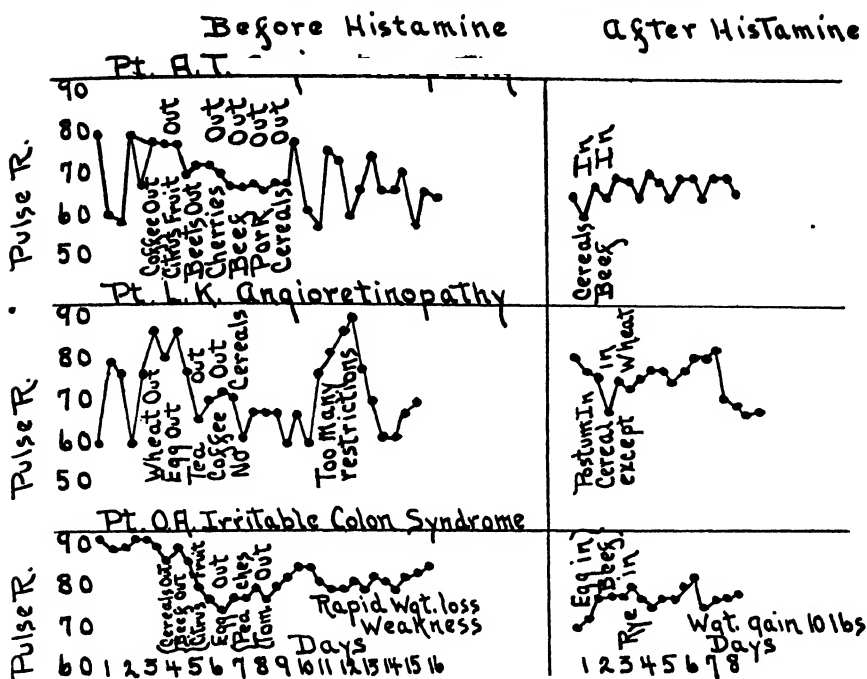


FIGURE 2. The epileptiform seizures and headache in BB were lessened, with no seizures for several weeks while eliminating the foods and with very little change in pulse rate before and after.

However, the acceleration is not completely eradicated when selected food allergens are still ingested. It is worth pointing out also that anti-histamine agents do not cure these people, which is a strong argument against histamine's being the sole factor.

The practical application of this interesting study is beset with many baffling problems. It is naturally dependent on an intact cardiovascular apparatus which is not being influenced by extremes of emotional excitement, physical exertion, febrile illness, or marked metabolic derangements.

The normal pulse is remarkably constant when not insulted by allergens. This is shown in FIGURES 4 and 5—the first from Coca's book and the next on two hard-working individuals, one age 28 and the other age 61, and on two people of my own acquaintance who have no complaints and no personal or family history of familial allergy. It is also shown on these tables that





the pulse is not stable when specific irritants are ingested. No restriction of activity was imposed on the people whose records are graphically illustrated.

This method may also be used in diseased hearts. I mention this only to show that, with the conduction apparatus severed, a response still occurs. This is illustrated by the influence on the apex rate of auricular fibrillation in two individuals whose fibrillation was on the basis indicated in FIGURE 6.

There is then a definite stability of the pulse rate in food allergic persons when the irritants are avoided. This was demonstrated by Coca on sixty-

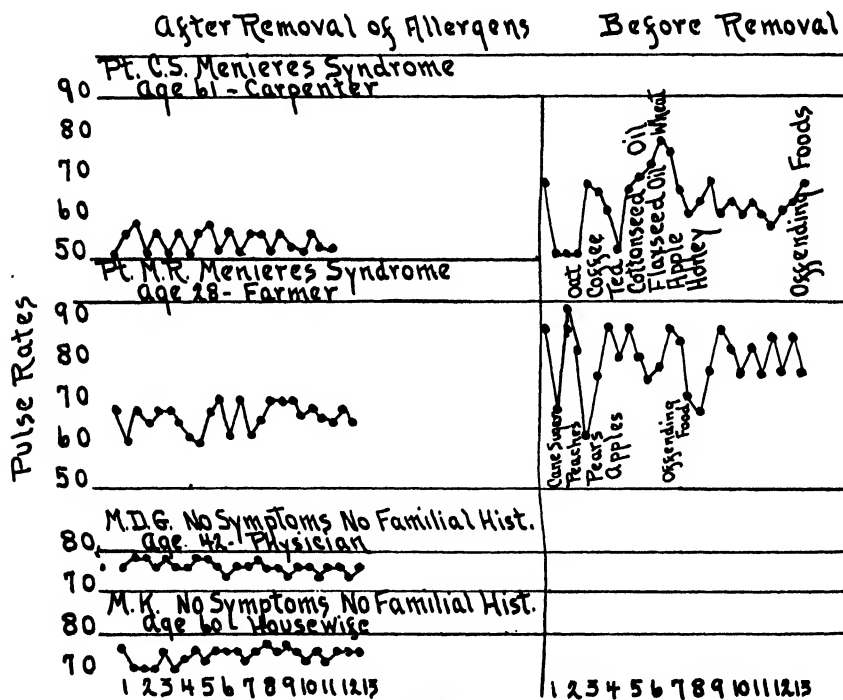


FIGURE 5.

two cases in whom he had successfully removed the allergenic foods. I should like to corroborate this with one hundred and six cases of my own who now not only have pulse stability but also have good health. These studies have naturally excited curiosity on the part of our own medical group. For a period of time, all patients, regardless of complaints, except pregnancies and traumatic cases, had pulse checks in our clinic, and thirty of those who were apparently the most co-operative recorded their heart rate after each meal, with the results shown in TABLE 7. The 60 per cent incidence leaves a relatively small number with stable pulse. Even in those, the stability may be on an accelerated plane due to the fact that the average pulse rate is *much* lower than we formerly thought.

*Practical Application*

It is imperative that the problems which will be encountered by the physician attempting this study be thoroughly understood. The failure to gain results and discouragement on patient's and physician's part can be

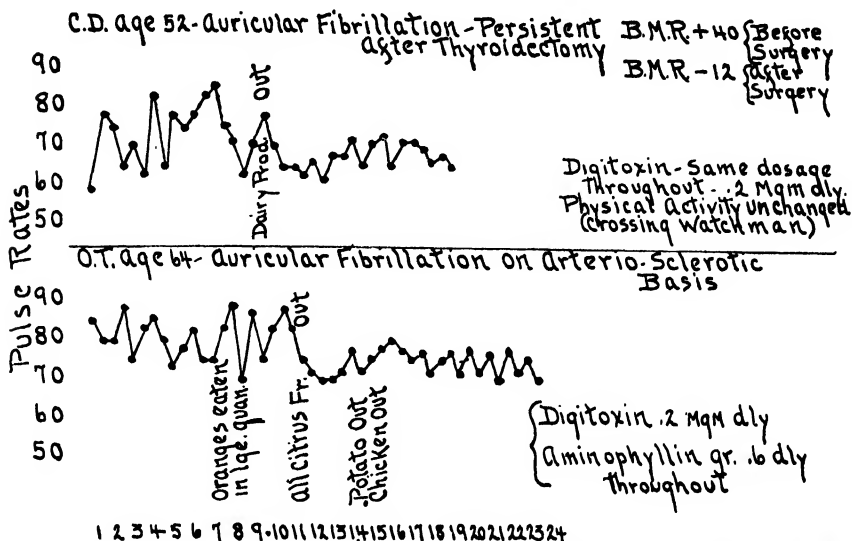


FIGURE 6. Note that medication was unchanged throughout the study. Physical activity was also unchanged before and after the removal of allergens. Fibrillation, however, is still persistent.

TABLE 7

No. of patients		Pulse over 80 in 2 readings
200		143 (71.5%)
No. of patients	No. with pulse variability p.c. of over 12	No. with personal and familial history suggesting idioblapsis
30	18 (60%)	16 (53.3%)

This indicates food allergy in about seventy per cent of the two hundred, except that diseased hearts were included. Of the thirty taken at random, regardless of complaints, but without known heart diseases or fever, food allergy is suggested in 60 per cent and is corroborated by history in 53.3 per cent.

avoided to a large extent by, first, a careful evaluation of the patient and, second, a very careful understanding of the method of interpretation.

The initial questions to be considered with the presenting patient are primarily three in number. These are indicted in TABLE 8. The first of these is best answered by having the patient, following his first office visit, carefully fill out the questionnaire reproduced in TABLE 9. This can be followed up and in most cases can be combined with a diagnostic résumé by giving him a trial diet (TABLE 10). This is a heterogeneous grouping of

common foods used only in the hope of demonstrating instability in the pulse rate with their ingestion. Explicit instructions are indicated not only for the recording of the pulse rates but also to gain complete patient co-operation. The necessity for the original use of enamelware or glassware rather than aluminum is indicated by FIGURE 7 (taken from Coca). This method can, in a large measure, determine the advisability of continuing further investigation. It especially does away with the need of spending much time in oral questioning and conference.

If the decision is such that further investigation is indicated, recourse may be had to one of two methods: (1) the patient may have a "free" diet,

TABLE 8

- 
1. Is the patient a person of food-allergic constitution?
  2. Can the chief symptom or symptoms complained of be identified as food-allergic, or is the patient predisposed to them by the handicap of food-allergic pathology?
  3. What are the specific excitants of the food-allergic symptoms in the particular case?
- 

TABLE 9  
QUESTIONNAIRE

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NAME.....	AGE.....
ADDRESS.....	

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If you have had or are having the following, *place a check (✓) mark after the disorder indicated.*

If any member of your family (mother, father, uncle, children, grandparents) have had or are having these disorders *place an X after it.*

- |  |  |
|--|--|
| <ol style="list-style-type: none"> <li>1. Asthma</li> <li>2. Hayfever</li> <li>3. Hives</li> <li>4. Migraine (sick headache)</li> <li>5. Chronic indigestion</li> <li>6. Colitis or Diarrhea (chronic)</li> <li>7. Nervousness</li> <li>8. Insanity or nervous breakdown</li> <li>9. Chronic eczema</li> <li>10. Epilepsy</li> </ol> | <ol style="list-style-type: none"> <li>11. Deafness or severe dizziness</li> <li>12. Stammering or stuttering</li> <li>13. High blood pressure</li> <li>14. Diabetes</li> <li>15. Canker sores</li> <li>16. "Shingles"</li> <li>17. Gall bladder diseases</li> <li>18. Cancer</li> <li>19. Ulcers</li> </ol> |
|--|--|

At what age and under what circumstances did your present disorder start:

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with only the limitation of four or five foods at a feeding (this allows them a more pleasant method of determining their allergen); or (2) the patient may be given only one food at a meal (although of some discomfort to the hard-working patient, this presents an easier method of interpretation). The latter method is used by Coca. This admonition should be injected here: If the patient's symptoms are severe enough to cause him to co-operate, few problems are encountered. If they are not, it is useless to attempt to force the issue, because one is certain to meet with disappointment.

Can the chief symptom be identified as being due to food allergy? The

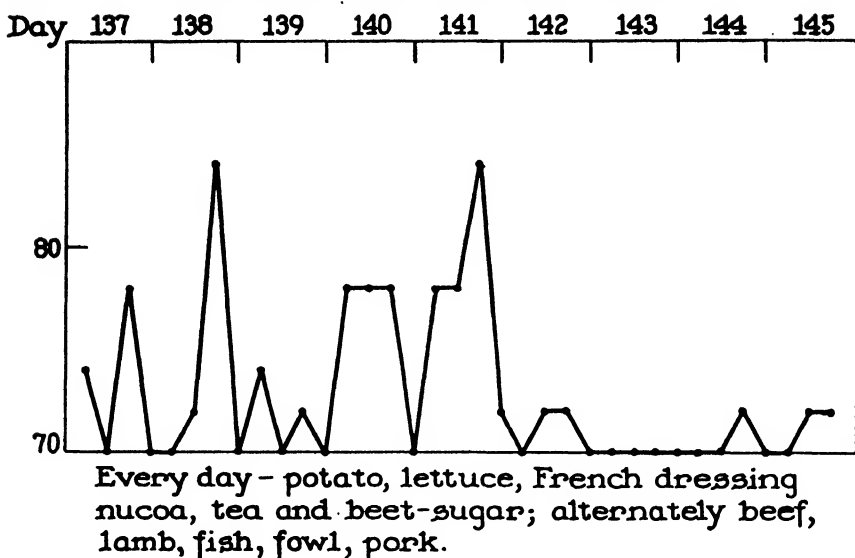
answer to this has, for practical purposes, already been gained by the foregoing questionnaire. The proof, of course, is in the disappearance of the symptoms when the offending substance is removed and, finally, to be therapeutically and scientifically correct, in the return of symptoms when the food is re-ingested. Complete confidence in the doctor is required to convince the new patient that the avoidance of certain foods will prevent the

TABLE 10  
PRELIMINARY TRIAL DIET

Pulse rate 4:00 P.M. Day before test starts	8:00 P.M.	9:00 P.M. Before arising 9:00 P.M. Day of test
<i>Menu</i>	<i>Pulse rates</i>	<i>Symptoms</i>
Rice Milk Beet sugar Grapefruit	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	
Beef Peas Potatoes Lettuce (no dressing) Pears (water packed)	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	
Rice Beet sugar Milk Butter Cheese Rye-Krisp	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	
<i>Second day</i>		<i>Pulse before arising</i>
Whole wheat bread Milk Butter Egg	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	
Beef Carrots Lettuce Beets	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	
Whole wheat bread Peas Chicken Grapefruit	Before $\frac{1}{2}$ hr. after 1 hr. after $1\frac{1}{2}$ hr. after	

development of other disabilities later in life. This, then, can only be done if one can prove that the present difficulties can be eliminated.

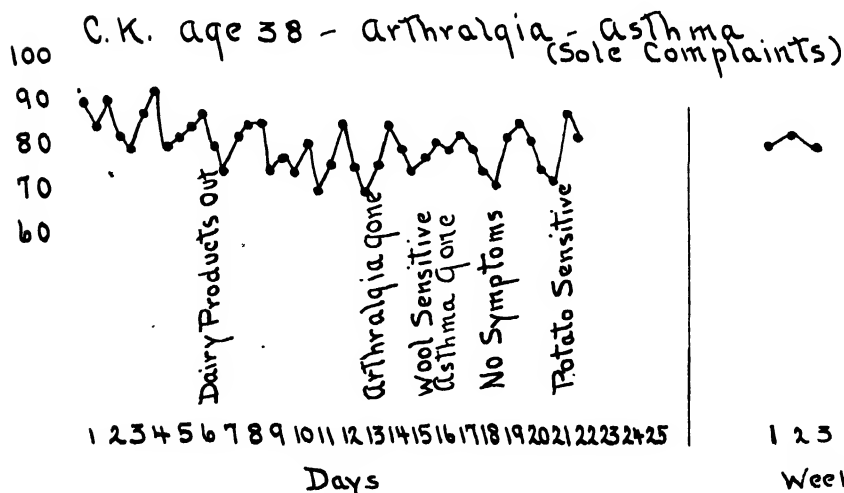
The specific excitants of the symptoms are primarily those that cause an increased heart rate. Yet, the disappearance of symptoms may occur with the elimination of only one or two of the foods that cause a heart-rate increase. This is illustrated in FIGURE 8. Note in the discussion that this patient experienced complete disappearance of arthralgia, which was



Cooking in aluminum  
and other metal

Cooking in enamel-  
ware

FIGURE 7.



Pt not interested after 3 weeks  
in eliminating potatoes since she  
had no subjective symptoms whatsoever  
from eating them

FIGURE 8.

associated with a tachycardia due to dairy products; yet the pulse is still unstable and the second food is ingested without complaints. Will symptoms appear in later life, as premature aging of certain organs and systems

TABLE 11  
FACTORS OTHER THAN FOOD-ALLERGENS THAT MAY CAUSE TACHYCARDIA

1. Smoking
2. Cathartics
3. Emotional disturbances
4. B-complex deficiency
5. Medications having specific effect on C.V. mechanism.
6. Metal cooking utensils
7. Dust
8. Inhalants (tooth powder, illuminating gas, coal smoke, *etc.*)

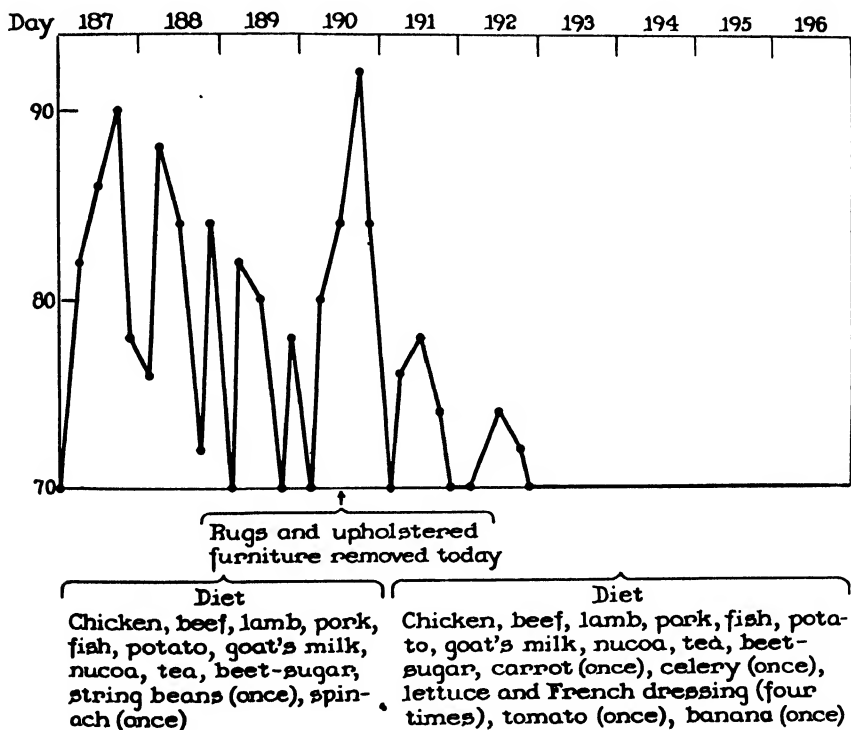


FIGURE 9

ensue? This question makes us carefully consider major and minor allergens. A discussion of these in detail will be given later.

As mentioned before, it has been shown that other factors than food are involved in a pulse response. These factors must be avoided at the time the diets are being followed. Most of them are listed in TABLE 11. The influence of dust is graphically illustrated in FIGURE 9 (from Coca).

I should like now to discuss in detail the individual difficulties encountered in interpretation and correlation of pulse-rate response. As I stated in the original review of Coca's book, these chapters invite the greatest controversial reaction. The discouragement attendant in those people exhibiting these problems has certainly forced abandonment of solution on many of my own patients. This in spite of personal enthusiasm for the theory. I can understand, however, why many physicians may remain cool to it, after perusing this chapter, with its numerous paragraphs as to why failure of identification may occur.

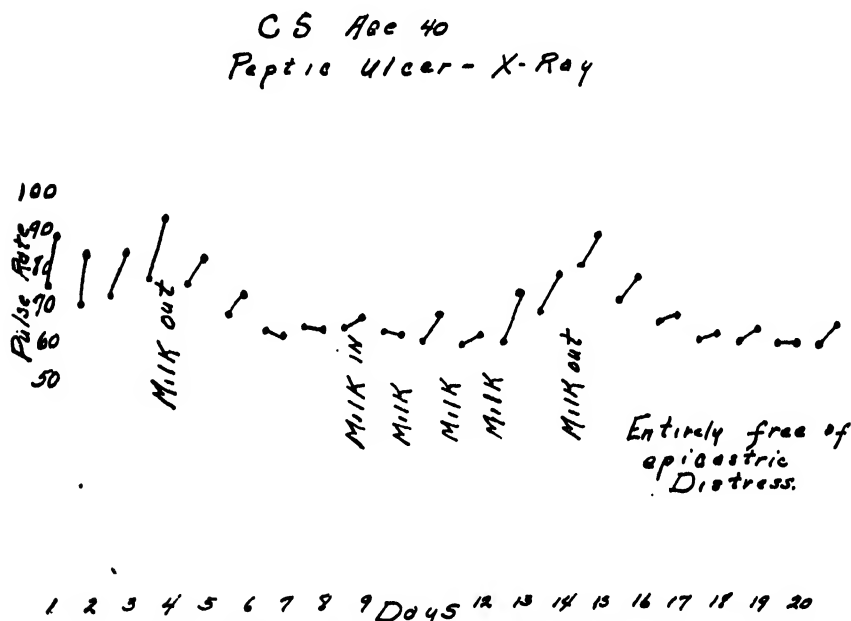


FIGURE 10. Note following primary elimination of milk that, beginning four days later, it was taken four successive days before any definite acceleration occurred, also that, in both instances, the tachycardia persisted for forty-eight hours after its elimination.

A concrete physiological explanation and scientific background for the specificity of the pulse-accelerating mechanism has many pitfalls, and its explanations are still debatable. The practical difficulties are also real. The careful study and application of reason as presented will enable those interested to solve most of the difficult cases. The problems are as follows:

(1) *The Latent Period of Temporary Lost Sensitivity.* This is best illustrated in FIGURE 10, graphically representing the temporarily lost sensitivity to milk. This phenomenon of course, is not new, having been mentioned many times by Vaughan.<sup>5</sup> This is another important differentiation from atopy, since loss of sensitivity here is probably due to natural desensitization rather than avoidance.

(2) *The Carry-Over Reaction or Recurrent Reaction.* This is common. I have encountered numerous examples of it as evidenced by the continued

elevation of pulse rate *prior* to succeeding meals when the breakfast has produced tachycardia.

These reactions are not too difficult to interpret. The removal of what are seemingly allergenic foods, however, may result in the elimination of some that are not. In that event, comfort may be impossible and the patient may prefer the chain of symptoms rather than a semi-starvation diet. I have found that a careful review of these cases in intervals of leisure is the only way to avoid the incrimination of food that is not allergenic.

(3) *Major and Minor Allergens.* This is one of the really discouraging phenomena of this therapeutic aid. In some instances, it makes for complete abandonment of the study on the part of the patient and physician.

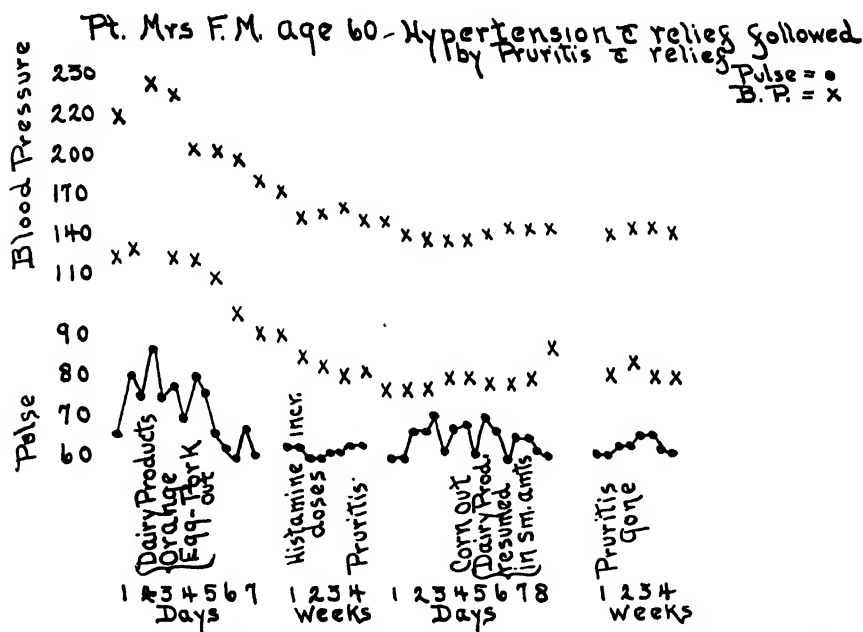


FIGURE 11. Histamine was given in this patient in the hope that dairy products might be reintroduced. After its discontinuance, corn, which had not been considered before, apparently became allergenic, with the perineum becoming the shock tissue.

In brief, the individual has dramatic relief of symptoms, only to be followed weeks later by a recurrence of the original symptoms or possibly a new chain of complaints. It is necessary that a frank discussion with the patient of this possibility be accomplished even before the problem is started. This particular phenomenon is graphically illustrated in charts 8, 9, 10, and 11 in Dr. Coca's book. In some instances, the patient has previously been acquainted with his intolerance to the food. At times, the newly detected allergen is botanically related to the previously indicted food, for example: the sensitivity to wheat and cereals was detected long before cane-sugar sensitivity was encountered.

The aspects of this phenomenon do offer some basis in fact for theoretical



explanation: that is, in many individuals, a non-specific tolerance to the minor allergens has been built up, due to repeated insults by the major allergens. If these major allergens have been eliminated, the production of "H"-body tolerance possibly becomes lessened. This may occur to the point where the body has no non-specific armor against the insults of the minor allergens. Then there occurs a renewal of the original symptoms or a new train of complaints as other organs become the shock tissue. This has been proved by Dr. Coca in his own cases, and I have an interesting corroboration of the same effect in a patient with hypertension. We then started histamine therapy, hoping for a better diet, which gave great aid, only to have a generalized pruritis develop. FIGURE 11 indicates this effect.

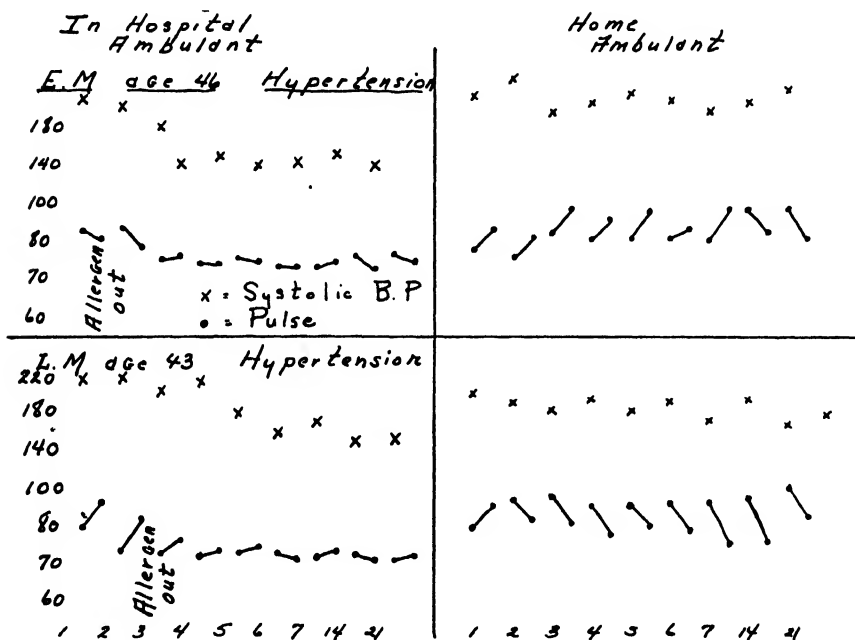


FIGURE 12.

(4) *The Sensitivity to a Large Number of Foods.* This must be proven before the assumption is accepted as correct. Briefly, one must be sure that inhalants and contacts are not entering into the picture. These, too, give an unstable pulse. In these patients with multiple food sensitivity, Coca is a strong exponent of sympathectomy. My patients to date have refused this operation, primarily because mystery of the rationale of interrupting the sympathetic chain is not only too great for them to understand but is too complex for me to explain adequately.

(5) *The Sensitivity to Unavoidable Inhalants Known and Unknown.* This can be illustrated by FIGURE 12 which refers to two patients with hypertension who were perfectly controlled, both in symptoms and in stability of pulse rate, when hospitalized without bed rest. When they were returned

to their homes, however, both symptoms and unstable pulse rates reappeared. In both of these individuals, all of the usual contacts and inhalants have been carefully ruled out.

Before going into the last general topic of this paper, it is well that we carefully evaluate the symptoms which may be ascribed to food allergy. Few of these conditions are regarded by most medical writers as having food allergy for their basis. The proof can only be sufficient if the criteria presented in TABLE 12 are met.

TABLE 12

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1. That there is a personal and familial history of a similar condition, or else those illnesses which are arbitrarily placed in the category shown in TABLE 2.
  2. That these symptoms have disappeared and reappeared as the foods involved were eliminated and reingested.
  3. That with the ingestion of these foods, an accelerated pulse is manifest if extraneous conditions are controlled.
- 
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TABLE 13

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Irritable colon syndrome.....	9
Pylorospasm.....	5
Peptic ulcer.....	2
Ulcerative colitis.....	1
Migraine.....	15
Menière's disease.....	3
Chronic rhinitis.....	2
Retinal angiopathy.....	3
Urticaria.....	6
Epileptiform seizures.....	5
Paroxysmal tachycardia.....	4
Angina pectoris (with EKG changes).....	2
Chronic asthmatic bronchitis.....	4
Acute mental depressions.....	2
Paralysis agitans.....	1
Multiple sclerosis*.....	2
Diabetes mellitus*.....	3
Emotional instability.....	5
Hypertension.....	18
Tinnitus.....	3
Myalgia.....	4
Pruritus ani-vulvae.....	5
Acne.....	2

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I have considered this last criterion because I have learned in several years of practice that the power of suggestion is a very potent weapon in many illnesses, not only those with a pathological basis but those without. It is my opinion that a mild hypnosis, combined with sustained interest on the part of the patient, relative to this new theory, may in itself ameliorate the symptoms.

### *The Results Obtained*

My personal experience has been limited to the symptoms listed in TABLE 13, which I believe to be food allergic by virtue of their disappearance

with food elimination or by the inability to gain a stable pulse when all influences were carefully controlled. In these latter, I regard my failures as being due to either misinterpretation of the trial diet or a sensitivity to such a multiplicity of foods that I could not maintain the patient on that diet. Special consideration of "multiple sclerosis" and "diabetes mellitus" must be given, inasmuch as these are the types of cases which must be followed over the course of several years before one is able to gain an understandable and unbiased opinion as to arrest or cure.

I must emphasize that I cannot assert at this time that all cases falling into the categories mentioned are due to food allergy, but I am becoming enthusiastic enough about the frequency in which they do to use the trial allergy survey on these individuals as a first resort rather than a last resort. I am also agreeably surprised to find that my interpretative ability increases geometrically with practice. As to the results obtained, with a brief discussion as to failures as well as successes, I repeat again that, for several reasons, one must always consider the extent of disability of the patients involved.

(1) Is the patient of relatively stable personality? If not, one always runs the risk of making a "chronic pulse counter" of the individual, with a natural fixation of a cardiac neurosis. The layman associates pulse rate and myocardial reserve as synonymous and parallel situations. Certain individuals have a vasomotor instability which would exist without food in a hermetically sealed room.

(2) Is the patient willing to give up enough of his daily pursuit of pleasure in food ingestion to follow carefully the prescribed program?

(3) Is the patient intelligent enough to realize that no cure is being promised until well after the analysis has started?

(4) If the case is proven to be the result of dietary indiscretion, is the patient willing to forego the particular food or foods involved? Many patients will, during the trial period, lose considerable weight due to the loss of "glamour" in the preparation and serving of the foods and to the loss of extra cellular fluid. These are rather feeble excuses for failures; yet they all exist. I must admit a growing feeling on my part that most people with allergy are emotionally more unstable than those without. Whether the allergy is the cause or the result, I do not know.

Hypertension presents the most interesting possibility, since it may be argued with a certain amount of scientific reasoning that the renal vascular apparatus may well be shock tissue and because it accounts directly and indirectly for such a high percentage of deaths in this country. Coca postulates as follows, following Goldblatt's theory, to a large extent:

(1) "*That ischaemia limited to the kidneys [author's italics] may be the initial condition in the pathogenesis of the hypertension that is associated with nephrosclerosis.* If this be true, then renal ischaemia, no matter how produced, should be followed by elevation of blood pressure."

(2) "Hypertension without or with disturbance of renal function, resembling in this respect the benign and malignant types, respectively, in man, can be produced by varying the degree of constriction of the renal arteries."

(3) "The kidney was assumed to be an independent allergic shock organ."

(4) "An hitherto unexplored physical factor in the causation of disease was assumed to be brought into play through the allergic reaction within the kidney. This factor is merely the increased subcapsular pressure caused by the allergic edema. It is easily conceivable that pressure developed in that way could retard the renal circulation sufficiently to provide the essential condition of the Goldblatt experiment. That condition is known to be provided when pressure is applied to the organ externally."

It is also conceivable that this physical factor of allergic subcapsular pressure may play a similar role in the etiology of diseases known to be due to a disturbance of the internal secretion of other organs.

TABLE 14

SIX CASES OF HYPERTENSION TREATED WITH THE METHOD OF PULSE-CONTROLLED TRIAL DIET

Case	Sex	Age	Blood pressure				Pulse maxima		Allergens
			previous to treatment		at termination of treatment		before treatment	after treatment	
			S	D	S	D			
1	F	63	198	120	124 118	72(3-2) 70(5-25)	98	72	Cereals, cane sugar Beef, pork, milk, nuts, potato, orange, banana, berries
2	M	32	160	110	122 142	84(12-31) 88(2-3)	92	76	Tobacco and other inhalants
3	M	50	160	100	128	88(2-3)	100	76	Cereals, cane sugar, potato, citrus fruit, pineapple, date
4	M	38	150	90	110	70(4-2)	94	78	Cereals, cane sugar, prune fam., squash fam., cabbage fam., tobacco, alcoholic beverages
5	M	40	134	90	106 112 126	74(4-27) 74(5-21) 72(6-5)	116	66	Cereals, coffee, tobacco
6	M	55	145	98	126 74		78	68	

It was pointed out that "if the renal vessels have been permanently narrowed by a chronic inflammatory process following infections (glomerulonephritis), or by the secondary sclerosis of malignant hypertension, this irreversible cause of hypertension would persist after the elimination of any existing food-allergy."

These postulates seem reasonable—certainly as reasonable as any of the theories regarding hypertension, including that of a specific rennin activator. Coca reports the cases illustrated in TABLE 14, with their allergens as described. I have had the opportunity to follow for one year and for six months, respectively, the individuals shown in TABLES 15 and 15a. These have remained very well controlled from the standpoint of objective findings as well as subjective symptoms.

It is also of interest to note that, with the falling pressure, none of these

patients has had evidences of impaired coronary circulation, which so often occurs when so-called specific medication to lower the pressure has been used.

These cases offer an interesting corollary to the success which Dr. Kempner of Duke University has achieved. With his rice and fruit juice diet, he has had good results in about sixty per cent of his cases. Certainly, it is within the realm of possibility that in his cases the elimination of the food allergen is responsible.

TABLE 15

Name	Age	Previous B.P. (6 or more readings average)		Present B.P. (10 or more readings average)		Allergens	No. of months followed
		S	D	S	D		
Mrs. P. M. ....	52	190	110	140	86	Beef, peas, str. beans, tomatoes, spinach	16
Mrs. S. R. ....	64	220	130	160	90	Eggs, celery, citrus fruit, apples	15
Mrs. M. M. ....	58	190	110	150	84	potatoes, beef, peas, str. beans	15
E. P. ....	40	166	100	142	84	citrus fruit, cane sugar, fish	15
Mrs. J. F. ....	47	180	120	146	80	eggs, pork, coffee, choco- late, chicken	15
J. D. ....	38	158	100	130	76	chocolate	15

TABLE 15a  
*Patients Followed 6 Months Minimum*

No. of patients	Average diastolic pressure before allergens removed	Average diastolic pressure after allergens removed
18	102	86
Total no. of hypertensives treated	Those now with diastolic B.P. below 100	Percentage
34	22	64/7

Until, however, actual food sensitization can be given the laboratory animal and a careful and accurate measurement of blood-flow through the kidneys can be ascertained before and after the allergic insult, all theoretical considerations of the exact mechanism involved will of necessity remain in the realm of conjecture. One is still forced into the position of stating, "My theory is as good as yours until proven otherwise."

Coca believes, and I certainly agree, that if kidney damage is such that nitrogen and dye retention has already occurred, there is little use to hope that regeneration of destroyed cortical substance can be accomplished.

Even in such patients, however, I have had the gratifying experience, in two cases, of having laboratory findings change as well as clinical symptoms. That some of the damage was transitory, due to vascular spasm or fluid pressure, is my only hypothesis. TABLE 16 illustrates this point. It is my feeling that Coca's theory can be substantiated in a rather high number of hypertensives. This only when the physician, as well as the patient, is willing to subject himself to the tedious processes described.

That food allergy will cause arterial spasm is an established fact. This statement is made in light of two illustrative cases which I saw in consultation with an ophthalmologist. These two patients suffered rather sudden loss of vision, both showing marked retinal angiopathy, one associated with

TABLE 16

Name	B.P.	Urine	Chemistry	B.P.	Urine	Chemistry
		(before allergens out)			(after allergens out)	
Mrs. E. L., age 36. Dizziness, head- ache. Exertional: dyspnoea, heart- conscious	250/140	Sp. gr. 1004	NPN—48	160/88	Sp. gr. 1016	NPN—32
	230/136	Alb—Tr Micro 3-4 hyaline 1-2 granular HPF	Urea N—30 P.S.P. 1 hr.—20% 2 hr.—22%	158/84 158/82	Alb—0 Micro Amorphurates only Only occasional	Urea—18 P.S.P. $\tau$ 1 hr.—40% 2 hr.—16% headache now
Mrs. S. R., age 62. "Palsy," dizzi- ness. Exertional: palpitation, an- gina, dyspnoea	210/130	Sp. gr. 1006	NPN—52	170/90	Sp. gr. 1014	NPN—40
	220/134	Alb—+ Micro 7-8 hyaline 4-5 granular HPF	Urea N—32 P.S.P. 1 hr.—28 2 hr.—18	172/88 168/88	Alb—spt Micro 1-2 hyaline HPF Dyspnoea only—and with marked exertion	Urea N—20 P.S.P. J 1 hr.—40 2 hr.—20

TABLE 17

15 CONSECUTIVE PTS.  $\bar{c}$  GASTRO-INTESTINAL COMPLAINTS

X-ray exam: judged neg. for organic pathology.....	8
X-ray exam or clinical and lab. exam. judged negative for organic pathology.....	11
Positive x-ray and clinical findings for organic disease.....	4
Number of patients well after elimination of allergens.....	10
Percentage of functional cases cured without medication.....	90.9%

a hypertension, the other with a normal blood pressure. They were both hospitalized for careful study and were both given multiple histamine doses in glucose saline. Their objective and subjective findings cleared almost immediately. This was followed by a careful study of possible offending foods, and after six months both patients, following elimination of the foods involved, had normal vision and normal ophthalmological findings.

Of those patients with symptoms referable to the gastrointestinal tract, it is seemingly becoming more and more apparent that the so-called "irritable colon syndrome" is in reality an allergic expression on the part of the digestive apparatus. TABLE 17 shows the consecutive number of cases seen with gastrointestinal symptoms, the laboratory findings, and the results. This is not a large series, but it must be remembered that, in private practice, a diversified group of people are seen and a complete

diagnostic survey to exclude all organic possibilities is sometimes difficult to accomplish because of monetary consideration on the patient's part. Naturally, it is within the realm of possibility, as Coca has suggested to me in personal communication, that all disordered functions of the digestive system may have allergy as their original basis.

Diseases of the nervous system, as previously illustrated, especially those in which we have no other readily available etiological explanation, certainly open the door in a small way into a fascinating and constructive train of thought. My personal experience with multiple sclerosis is not of sufficiently long standing even to be acceptable; yet in two patients, followed for six months, there has been a complete arrest of symptoms. Epileptiform seizures, as indicated by Coca, have been greatly lessened in severity and frequency and in some cases abolished. My experience so far is not as completely satisfactory as his. The best results I have obtained are in those individuals in whom the aura of these attacks is headache. I am quick to

TABLE 18

<i>Name</i>	<i>Age</i>	<i>Average freq. of attacks</i>	<i>Grand mal</i>	<i>Frequency</i>	<i>Medication as before</i>
B. B. ....	24	3 times weekly	+	3 times wkly.	+
L. C. ....	19	5 times yearly	+	2 times in 15 mos.	+
L. N. ....	34	2 times monthly	+	1 time in 4 mos. Known diet. indis- cretion	0
M. M. ....	62	2 times monthly	+	1 time in 6 mos.	0
C. T. ....	64	3 times weekly	+	2 times in 10 mos.	$\frac{1}{2}$ dosage Dilantin S.
L. S. ....	26	3 times daily	0	3 times daily	+
E. P. ....	58	4 times monthly	0	1 time in 6 wks.	No medication

admit, however, that I have had no sympathectomies performed. TABLE 18 shows the epileptics seen by me, with the results obtained.

Coca presents some very definite investigative figures on individuals with frank psychosis. To my knowledge, no concerted efforts have been made to follow up these acutely interesting conjectures. Certainly, the results accomplished in those people with emotional instability, tinnitus, and Meniers Syndrome and, in one case only, the relief from the distressing symptoms of paralysis agitans make the investigator rather enthusiastic in his belief that the central nervous system may also be shock tissue.

The sensitization of the cardiovascular apparatus to allergens, in the opinion of Coca and myself, is well supported. Whether this be through the mechanism of the autonomic nervous system or by direct action on both the conduction system and the arterial caliber is difficult to evaluate. I refer primarily to angina pectoris, paroxysmal tachycardia, and neuro-circulatory asthenia.

The inheritance factor of idioblastis is unquestioned if one is willing to "go along" with Coca in the acceptance of the large number of symptoms which he and I regard as arising from idioblastis. Statistical support for

his contention is contained in his careful survey of school children in his own community, and, to my mind, it is well supported by the questionnaires which we in our own group utilized. Similar interesting observations suggesting the 100 per cent incidence of a familial background have been recorded by others.

A summary in any paper having for its main thesis a group of another's postulates is difficult. However, I should like to reiterate those points which I believe warrant serious and concentrated study on the part of all interested physicians. They are as follows:

(1) There is apparently a fifth category of allergic illnesses, named by Coca, "idioblapsis."

(2) This category has a definite symptomatology.

(3) The allergenic material or food causes an acceleration of the pulse, and a careful study of the pulse rate is the only accurate means of identifying the offending articles.

(4) Failure of identification is based primarily on the physician's inability to interpret the effect of the latent period, the recurrent reaction, the differentiation of major and minor allergens, the depression of shock tissue with repeated insults, and, finally, the sensitivity to a large number of foods or unavoidable inhalants.

(5) A rather large group of symptoms referable to almost all of the bodily systems have been presented with statistical data to support the contentions that they are of allergic origin.

(6) The possibilities for further investigation are almost inconceivable in their scope, and further corroboration of this fascinating subject is earnestly sought.

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# FAMILIAL NONREAGINIC ALLERGY AS A PREDISPOSING CAUSE OF COMMON COLD

By Arthur P. Locke

*Zonite Products Corporation, New Brunswick, New Jersey*

This is a report of an investigation begun sixteen years ago at St. Luke's Hospital, Chicago, and carried from there to the Western Pennsylvania Hospital, Pittsburgh, and to Stephens College, Columbia, Missouri. It began as a search for an answer to the question: Is there any way of telling, beforehand, when a group of animals is given an infection of borderline virulence, which animals probably will live and which probably will die? The answer to this question led the way to a second: Is there any way of telling, beforehand, when a group of persons has been exposed to common cold, which persons probably will develop common cold and which will not?

This second question is our objective. But, first, what is known about susceptibility to infection, in animals? A great deal was known, even sixteen years ago. Many factors had been found that actively lower resistance to infection. Among them were shock, and injury producing shock, poisoning, starvation, endocrine deficiency, and exhaustion.

During anaphylactic shock in dogs, Boone, Chase, and Brink<sup>1</sup> had observed a lessened resistance to invasion from foci in the intestines. Blood cultures persisting for hours beyond the time normally observed had been found by Weisberger<sup>2</sup> and by Burn, Chandler, and Hartshorn,<sup>3</sup> in rabbits injected intravenously with streptococci, during anaphylactic shock. Robertson and associates<sup>4</sup> had shown that the resistance to pneumococcal invasion can be lowered by morphine. We<sup>5</sup> had found that morphine in massive dosage can be so effective an opener of the doorway to infection that infections can be established in rabbits with a type of pneumococcus not normally invasive. Vitamin C depletion, to the point of severe scurvy, was found by McCullough<sup>6</sup> to have a related effect in the guinea pig. Pickrell<sup>7</sup> found that alcohol in high dosage so lowered the capacity for resistance as to lessen even the protection given by antisera.

These different ways of lowering resistance had one factor in common. They interfered with the ability of the animal to function efficiently. Particularly, they interfered with the ability to keep warm. It is common knowledge what shock can do to body temperature. Morphine and ether, in amounts sufficient to lower resistance to infection, also lower the body temperature.<sup>4,7</sup> This tell-tale indication, that lowered resistance to infection may go hand in hand with lowered resistance to chilling, and to other emergencies with which life can be confronted, was explored further.

A method was worked out for determining ability to warm up after chilling.<sup>8</sup> Rabbits were used. They were chilled in cool water until the rectal temperature had fallen to 96. Then they were dried and allowed to warm up spontaneously. The unit adopted was the time required for a rectal temperature rise from 96 to 99—the *warming time*. The temperature rise was plotted on chart paper as in the examples in FIGURES 1-4.

TABLE 1 compares the response of two rabbits to a test chilling and to a later test of ability to dispose of intravenously injected pneumococci. The rabbit that had made the faster recovery from chilling had a blood culture of 2 pneumococci per ml. one hour after injection and was negative at the end of three hours. No fever developed, and no weight was lost. The rabbit survived. The rabbit that had made the slower recovery from chilling was slower also at the task of disposing of injected pneumococci. The count had decreased only to 44 at the end of the first hour. Multiplication had begun and the count had become increased to 244 at 3 hours. Fever developed and death occurred within 48 hours.

A larger series was studied to determine whether this parallel between the rabbit's ability to respond quickly to the demands of chilling and its

TABLE 1

RATE OF TEMPERATURE RECOVERY AFTER A TEST CHILLING, AND RATE OF REMOVAL FROM THE BLOOD STREAM OF INTRAVENOUSLY INJECTED TYPE I PNEUMOCOCCI

<i>Minutes required for spontaneous rise of rectal temperature, after chilling, from 96 F to 99</i>	<i>Number of pneumococci demonstrable, per ml. of blood, at the indicated hour</i>		
	0	1	3
35	114	2	0
60	100	44	244

TABLE 2

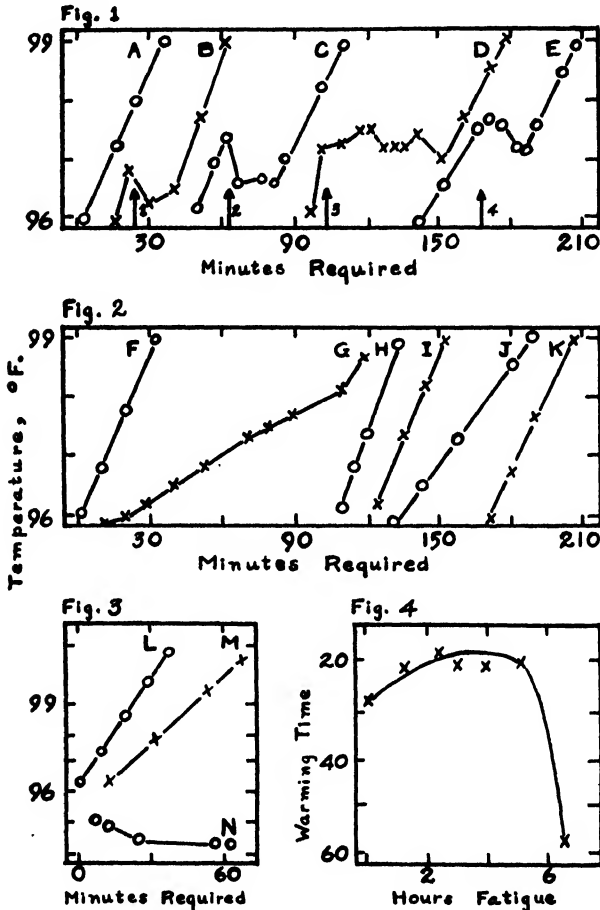
PER CENT SURVIVAL FOLLOWING INTRAVENOUS INJECTION OF TYPE I PNEUMOCOCCI

<i>Warming time at time of infection</i>	<i>Number of rabbits studied</i>	<i>Per cent surviving</i>
Less than 35.....	11	82
35 to 45.....	21	47
More than 45.....	9	0

ability to respond quickly to the demands of infection was a matter of chance or an indication of general principle. TABLE 2 summarizes a series of comparisons of warming time and resistance following intravenous infection in 41 rabbits. A sufficient number of virulent, type I pneumococci was injected to produce one death, roughly, in every two. A total of 19, out of the 41, survived. The rabbits had been tested for their reaction to chilling two days or longer before their test for reaction to infection. Eighty-two per cent of those which had been found to have a fast warming time survived, as compared with zero per cent for those which had been found to have a slow warming time. The rabbits which lived were those which disposed of their infections before those infections could get an overwhelming start. The rabbits which died were those so slow in disposing of their infections that overwhelming multiplication set in before the job of getting rid of the

bacteria could be completed. Also, this difference in speed of disposing of a test infection was paralleled by the earlier determined difference in speed of temperature recovery after chilling.

FIGURE 1 shows what happens to the warming time during shock. Rabbit A is the control. Shock was produced in rabbit B by an injection of his-



FIGURES 1-4.

tamine<sup>8</sup> at the time marked by the arrow, following which temperature recovery stopped for 20 minutes. Anaphylactic shock had a related effect in rabbit C. Pitressin produced a similar transient cessation of temperature rise in rabbit D.

This action of anti-diuretic pitressin is of special significance. Nedzel has reported that injections of pitressin can lower resistance to infections of a type associated with endocarditis.<sup>20</sup> Coca<sup>17</sup> has called attention to the rapid increase of weight which sometimes is noticed at the onset of allergic

headache and the equally rapid loss of weight as the attack passes off and states that "Some observers have reported that the loss of weight has been associated with an increase in the excretion of urine. . . ." Water retention, followed by diuresis, also comes into the common cold picture.

F, G, and H, in FIGURE 2, describe the warming time before, during, and after action of morphine in depressant dosage.<sup>8</sup> Rabbits M and N were subjected to a six-day fast. M was a healthy rabbit, free of discernible infection. N had infected wounds. The warming time is lengthened by prolonged starvation, and the effect is more serious in an infected rabbit than in one free of infection.

The first effect of progressive fatigue, as shown in FIGURE 4, is to shorten the warming time. It produces stimulation. After exhaustion, the warming time lengthens to an hour or more. Fatigue brings on exhaustion only slowly in healthy rabbits but rapidly in rabbits harboring infection. The sensitivity to shock also is increased when the warming time has been excessively shortened by fatigue or other stimulation.<sup>9</sup>

The 33 rabbits in TABLE 3 were given a shock injection of histamine by vein which killed approximately one-half.<sup>9</sup> Eighty per cent of those with

TABLE 3  
WARMING TIME AND SUSCEPTIBILITY TO HISTAMINE SHOCK

Warming time at moment of shock injection	Number of rabbits tested	Per cent developing fatal shock
16 to 21.....	10	80
22 to 30.....	17	47
More than 33.....	6	0

warming times between 16 and 21 developed fatal shock, as compared with zero per cent for those with warming times longer than 33. It can be seen, with this observation, where the facts are leading. The rabbits with a slow warming time are unable to *keep up* with the demands of infection. The rabbits with too fast a warming time are unable to withstand shock. Natural infection requires resistance not only against the growth of the invading organisms but, also, against those products of their growth which cause effects related to shock. This means that the warming time range associated with most effective resistance to prolonged infection, in the rabbit, must be neither too slow nor too fast, but in between.

The rabbits in TABLE 4 received an injection of type I pneumococci into the skin, creating a focus in which the pneumococci could multiply out of direct reach of the defenses available to the blood stream.<sup>9</sup> Prolonged resistance was called for. Only 6 out of the 50 were able to survive. These 6 which survived had warming times that were neither too fast nor too slow, but in between. Our first question had been answered. One *can* tell, beforehand, out of a series of rabbits given an infection of borderline virulence, which ones have the greatest chance for survival and which ones have the least.

Before the investigation could be extended from rabbits to man, three further questions had to be answered. (1) Does common cold represent an infection of borderline virulence that can be utilized as an index of resistance in the way that resistance to pneumococcal infection was used in the rabbit? (2) Do differences exist between different individuals, in susceptibility to common cold? (3) Can tests be made on human subjects giving the information that is given by the warming time test for the rabbit?

The common cold is considered, by the writer, to be an infection of a community. The reason is this: when common cold is viewed for its effect on a community, a definite, reproducible pattern can be seen.<sup>10</sup> When it is viewed for its varied effect on the individual persons making up that community, it is neither definite nor reproducible. The common cold is a clinical entity, running a characteristic course<sup>11</sup> in the community as a whole and having certain, well-known common effects on the individual at onset, but it varies from individual to individual in the sum total of pathogens actively involved and in the complexity of the infection set up.

TABLE 4

PER CENT SURVIVAL FOLLOWING INTRADERMAL INFECTION WITH TYPE I PNEUMOCOCCI

<i>Warming time at time of infection</i>	<i>Number of rabbits studied</i>	<i>Per cent surviving</i>
16 to 20.....	3	0
21 to 29.....	11	9
30 to 35.....	14	29
34 to 40.....	9	11
More than 40.....	13	0

Marked differences are known to exist in susceptibility to common cold. Paul and Freese<sup>12</sup> saw this, in a study made in Spitzbergen. An outbreak of common cold had followed the arrival of a boat. It spread in the characteristic way until it had involved almost all the people there. However, a small number who, in the words of Paul and Freese "were constantly exposed and who were under close scrutiny failed to show susceptibility. . . ." The proportion of persons failing to show susceptibility was about 1 in 10. Brown,<sup>13</sup> in a more recent report from the Aleutian Islands, found that about one in eight, in his group, remained free of common cold. That is about the proportion found in our studies in Pennsylvania<sup>14,16</sup> and Missouri<sup>11,16</sup> and by Coca in New Jersey.<sup>17</sup>

The development of a test giving information on human subjects, like that given by the warming-time test for the rabbit, presented difficulties that required five years for solution.

One of the tests evolved measured the reaction to the respiratory demands of exercise.<sup>14</sup> A preliminary medical examination was given, and record was made of the height, weight, and vital capacity. Work was begun with the arms and legs, on an exercising machine. During the work interval, a record was kept of the breathing and use of oxygen. Following com-

pletion of a satisfactory test, the subject was asked to report weekly as to freedom from common cold. At the end of the year, the tests and the colds record were compared. The subjects who had had colds most frequently were, for the most part, those who had responded to the test like the too fast and too slow warming rabbits. The subjects with the fewest colds had made an in-between type of response.<sup>14</sup> Two additional groups were studied to make sure that this relationship was not accidental. The findings are summarized in TABLE 5.

Seventy to 82 per cent of the persons with responses to the exercise test, like those found the year before for those with fewest colds, had a better than average colds record. A better than average colds record was made by only zero to two per cent of those with responses clearly divergent from the range found to be optimum the year before.<sup>14</sup>

A further test measured the reaction to inhaled carbon dioxide.<sup>15</sup> After a preliminary examination and measurement of the height and weight, the subject was asked to relax in a reclining position. The blood pressure and

TABLE 5  
RESPONSE TO EXERCISE TEST AND SUBSEQUENT COLDS RECORD

Response to test	Year	Number of persons	Per cent with a colds record	
			Better than average	Worse than average
Optimum*	1936-7	33	70	0
	1937-8	21	82	0
Clearly divergent	1936-7	58	2	45
	1937-8	22	0	32

\* As indicated by a preliminary series, studied through 1935-6. Borderline groups omitted. Total number of subjects: 1935-6, 87; 1936-7, 155; 1937-8, 68.

pulse were taken and a mask was fitted over the face and connected to a device tracing the frequency and depth of breathing. This tracing was examined and was seen to be within the normal range for the observed height and weight while breathing ordinary air. Then a valve was turned, supplying air containing five per cent of carbon dioxide. In some of the subjects, the added carbon dioxide only slightly increased the rate and depth of breathing. In others, the carbon dioxide caused the breathing to become greatly increased. Still others made an intensity of response that was in between.

A second test was made, one to two weeks later. Each subject was questioned weekly as to freedom from common cold. At the end of the year, the tests and the colds records were compared. The subjects who had had colds most frequently were those, for the most part, who had made only a slight or else a very pronounced response to the carbon dioxide. The subjects with the fewest colds had made a response of intermediate intensity. A second series was studied the following year.

TABLE 6 summarizes the observations for the second year. Sixty-five persons had been tested and had faithfully reported their common cold status. Of these, only 16 had made the response to carbon dioxide which had been found, during the preceding year, to be associated with lowest incidence of common cold. Seventy-five per cent of this group of 16 remained cold-free for intervals of 4 months or longer. The average number of colds was 1.6. In the contrasted group, who made the responses to

TABLE 6  
RESPONSE TO CO<sub>2</sub> TEST AND SUBSEQUENT COLDS RECORD, 1939-40

<i>Response to CO<sub>2</sub> per cent divergence from optimum*</i>	<i>Number of persons</i>	<i>% remaining cold-free for 4 months or longer</i>	<i>Average number of colds per person</i>
0 to 5.....	16	75	1.6
6 to 10.....	30	40	2.7
More than 10.....	19	21	3.7

\* As indicated by a preliminary series, studied through 1938-9.

Fig. 5 SHOWING THAT RESISTIVE EFFICIENCY PARALLELS FUNCTIONAL EFFICIENCY

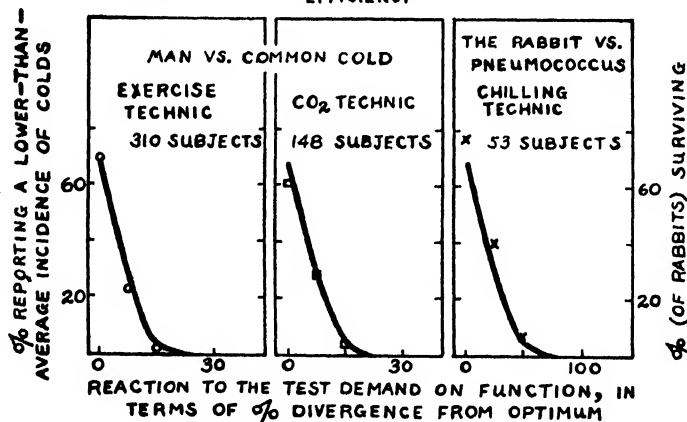


FIGURE 5.

carbon dioxide diverging most widely from optimum, only 21 per cent remained cold-free for 4 months or longer, and the average number of colds per person was 3.7.

If one takes the per cent divergence from optimum in the warming time as an index of decrease in functional efficiency for the rabbit, the per cent divergence from optimum in the responses made to the exercise test and to the carbon dioxide test as indices of decrease in functional efficiency for man, the number of colds as an index of resistive efficiency for man, and the per cent survival following pneumococcus infection as an index of resistive efficiency for the rabbit, a striking parallelism is apparent (FIGURE 5).

This parallelism suggests that common cold may become established most frequently in those of us who, like the too slow and too fast warming rabbits, are unable to maintain a continuously efficient defense against infections of borderline virulence.

What is the cause of this handicap? What possibly can happen to the large fraction of the population that is subject to common cold, that is sufficiently frequent and sufficiently severe to account for it?

Before our search for an answer to these questions had gone very far, a paper appeared by Coca<sup>18</sup> describing a type of allergic reaction to ingested food that met all of the test requirements. It affected a percentage of the population of about the same size as is affected by common cold. It was apt to affect its subjects frequently, so as to make them repeatedly vulnerable. It could be seen that, during a period, however brief, that the digestive tract might be reacting as a shock organ to an ingested allergen, the doorway might be thrown as widely open to common cold as it had been found to be opened to experimental infection in rabbits during periods of reaction to anaphylactic shock.

Coca had found that, when persons with a nonreaginic food allergy eat the food or foods that provoke the allergy, a sharp rise in pulse rate occurs which exceeds the rise produced by foods which do not provoke the allergy.<sup>19</sup> The persons giving this pathognomonic, pulse-rise indication of disturbed adjustment and impaired efficiency following the ingestion of a specifically trouble-making food were found to have histories, generally, of one or more of the following accessory indications, in recurring, cause-and-effect relationship with the food source: headache, hives, indigestion, diarrhea and constipation, canker sores, nervousness, and tiredness.

There are two striking things about this list of accessory symptoms. First of all, they are symptoms commonly and frequently experienced. Coca has found some degree of nonreaginic allergy in 75 to 80 per cent of the groups that he has examined. Anything affecting 75 to 80 per cent of us to an extent which invites common cold must be both common and frequently experienced. In this field of allergy and common cold, we must realize that we are looking for things that affect almost all of us—things that *are* common and frequent, but none the less important when properly considered. The second striking thing about this list is its length. Disturbed adjustment and impaired efficiency can be far-reaching, complex, and varied in final result.

Coca saw how closely the percentage of persons with nonreaginic allergy approaches the percentage susceptible to common cold. He had, shortly before, made a survey of the frequency of common cold among his associates and had, ready for study, a group of about 52 persons who had not had a cold for 3 years. Against this, he balanced a group of 51 with a history of at least one cold annually. The two groups were questioned for indications of the presence of nonreaginic allergy in themselves and in their families.

Ninety-two per cent of the colds-susceptible group had two or more indications of a background of nonreaginic allergy. Only 12 per cent of the nonsusceptible group was so affected. This striking difference showed



clearly the frequency with which symptoms indicative of nonreaginic allergy are found in persons subject to common cold and the infrequency with which they are found in persons not subject to common cold.

A confirmation of Coca's findings was obtained at Stephens College, Missouri.<sup>16</sup> Six hundred students were questioned there by an examiner trained by Dr. Coca but working under independent direction. The findings were checked against information secured from the parents and by a series of second interviews by a referee observer.

The findings of the questioner trained by Dr. Coca paralleled closely the findings obtained by the referee observer. Headache, recurring in a cause-and-effect relationship with food, was found in 57 to 67 per cent of the students questioned. Tiredness, nervousness, dizziness, constipation, indigestion, urticaria, and canker sores were found in 13 to 37 per cent. The average number of symptoms found, per student, was 3. Seventy-nine per cent of the students had 2 symptoms or more. This figure, 79 per cent was close to the figure of 75 to 80 per cent found by Coca in New Jersey.

TABLE 7

NUMBER OF COCA'S 11 SYMPTOMATIC INDICATIONS OF NONREAGINIC FOOD ALLERGY\* IN THE CHILDREN OF PARENTS WITH AND WITHOUT MORE THAN MINIMAL EVIDENCE OF SUCH HANDICAP

Number of parents	Number of children	Number of symptoms		Per cent of the children with two symptoms or more
		Father	Mother	
284	222	less than 2	less than 2	19
70	57	2 or more	less than 2	37
206	165	less than 2	2 or more	45
238	199	2 or more	2 or more	63

\* As reported by the parents both for themselves and the children.

The information obtained from the parents tended to be less complete than that obtained directly from the students, but did have the value of presenting that information against a background of equivalent information about themselves and the sisters and brothers (TABLE 7).

Passing downward, in TABLE 7, from a combination of father and mother, neither of whom had two or more indications of the presence of nonreaginic allergy to a combination of father and mother both of whom had two or more such indications, we also pass from a figure of 19 per cent for the children having 2 indications or more to a figure of 63 per cent. The significance of this observation is modified by the common origin of the figures compared, but not seriously so. It is exactly the type of relationship to be expected from Coca's classification of nonreaginic allergy as familial.

Each of the symptoms questioned for, when lined up with the number of colds developed during the following year, could be seen to be associated with a larger number of colds than was observed in the absence of symptoms (TABLE 8). The group with no symptoms, no hay fever or asthma and a response to the Flack test which was neither excessive nor subadequate,

had an average of one cold for the year. (The Flack test had been used in an exploratory way as an index of efficiency.) The corresponding group with two symptoms or more had an average of 1.8 colds for the year.

Addiction to smoking was found to be a factor further affecting the results. Both the effect of smoking and the effect of an excessive or subadequate response to the Flack test were confirmed in a further investigation the following year.<sup>11</sup> When the figures were sifted for these complicating factors, and when smoking and unfavorable response to the Flack test were given a weight of two symptoms each, a clear parallel was found between number of colds for the year and number of sources of allergic or other handicap. The group with the largest handicap had almost four times as many colds for the year as the group with smallest handicap.

TABLE 8  
SYMPTOMATOLOGY AND COLDS INCIDENCE; SUBJECTS FREE OF HAY FEVER AND ASTHMA

Symptom*	Flack test pulse rise between 6 and 24		Flack pulse rise less than 6 or more than 24	
	No. of persons	Avg. no. of colds	No. of persons	Avg. no. of colds
None presented.....	14	1.00 $\pm$ .17†	10	1.30 $\pm$ .27†
One only.....	31	1.06 $\pm$ .11	18	1.83 $\pm$ .16
More than one.....	155	1.80 $\pm$ .07	107	2.14 $\pm$ .10
Hives.....	57	1.9	42	2.6
Headache.....	128	1.8	88	2.3
Indigestion.....	42	1.9	34	2.3
Canker sores.....	75	1.7	59	2.2
Dizziness.....	35	1.9	26	2.5
Constipation.....	62	1.9	33	2.1
Nervousness.....	39	2.0	30	2.7
Tiredness.....	50	1.7	43	2.7

\* As reported by examiner I. G.

† The probable error, Peter's formula.

The probability would seem to be substantial that the entity described by Coca as familial nonreaginic allergy does exist, to an extent affecting somewhere near three-fourths of us and to a degree providing a trigger, touching off intervals of inability to cope with emergencies, that may be in the background of many disease pictures. Common cold is only one of many infections by pathogens of borderline virulence that may have a beginning or a recrudescence during an interval of food-allergic reaction.

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# THE ANTIALLERGIC ACTION OF SYMPATHECTOMY\*

By Arthur F. Coca

*Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York*

To the best of my knowledge, the operation of sympathectomy as a consciously antiallergic measure was first performed on myself by Dr. Laurence Miscall at my request. As I was aware, the operation had been successful in some of the cases in which it had been employed upon an empirical basis for the reduction of hypertension. I think my knowledge of that fact was the most persuasive consideration impelling me to the operating table, because I had become convinced of the primarily allergic nature of hypertension.

My own history illustrates very well the nature of the antiallergic action of the operation, as well as its limitations. Also, in general, it contributes to the very important practical conclusion that the maximal antiallergic effect is obtainable with the least transverse section of the main sympathetic chain that can be permanently maintained.

Through many years of trial and error, at first without, later with the use of the criterion of specific tachycardia, I had found myself clinically allergic to all available foods excepting beef. In ignorance of Stefansson's demonstration of the indispensibility of fat for a man's nutritional requirement on an exclusive diet of meat, I ate only lean beef and rapidly lost both weight and appetite.

TABLE 1 presents the more common of my allergenic foods in approximately the chronological order of their identification. For some months after they were first identified as allergens, the foods listed in the second and third columns could be eaten without symptoms at one or two-week intervals. None of these was being eaten when the foods in the fourth column began to cause symptoms. The latter are in heavy type to indicate that, shortly after daily subcutaneous injections of histamine were begun, all three of them could be restored to the diet without any reaction.

In May, 1942, Dr. Miscall performed the first stage of the sympathectomy (Crile). Three right lumbar ganglia and the right half of the celiac ganglion were removed, and 2.0 cc. of absolute alcohol were injected into the left half of the celiac ganglion. Three and one-half months later, the second stage of the Crile operation was carried out.

After the first operation, the following symptoms disappeared without need of any restriction of the diet: migraine, gastric pain, gastrointestinal bleeding, constipation, hemorrhoids, chronic rhinitis, and lapses of memory. There remained tiredness, neuralgia (occipital and sciatic), with occasional numbness of ulnar nerve distribution, and hypertension (up to 190/122) and, also, canker sores, heartburn, and mild conjunctivitis.

It is noteworthy that the residual symptoms were not perceptibly modified by the second operation. This observation caused me, thereafter, to recom-

\* Much of this material has been published in the *Annals of Allergy*, March-April, 1947, and in *Familial Nonreaginic Food Allergy*, Charles C. Thomas, publisher, Springfield, Ill.

mend only the most conservative procedure—removal of only three lumbar ganglia on one side. With one exception, all of my operative patients were so treated, the exceptional patient having had a left cervical sympathectomy.

Several months elapsed after the operations on myself and on the several patients who followed me before I realized the improbable and still inexplicable fact that sympathectomy abolishes not all-or-none of the sensitivities but, selectively and unpredictably, the sensitivity to *certain* of the allergenic foods.

TABLE 2 presents again the list of my food allergens. But, this time, the items in heavy type are those which, since the operation, have been eaten in unlimited quantity without any allergic reaction. It is seen that, whereas

TABLE 1\*

wheat	rice	potato	milk
pork	oat	peach-plum	fowl
lemon	tomato	fish	banana
corn	lettuce	onion	
sugar-cane	cabbage (fam.)	spinach	
sweet potato	chocolate	carrot	
apple	orange	beet	
	pea-bean	grapefruit	
	peanut	egg	

\* Showing the nonselective abolishment of the sensitivities to the three weak food allergens in patient A. F. C. under daily injections of histamine diphosphate. The items in heavy type could be eaten without allergic reaction during the period of the injections.

TABLE 2\*

wheat	rice	potato	milk
<b>pork</b>	oat	peach-plum	
lemon	<b>tomato</b>	<b>fish</b>	<b>fowl</b>
<b>corn</b>	lettuce	onion	
sweet potato	cabbage (fam.)	spinach	<b>banana</b>
apple	<b>chocolate</b>	carrot	
<b>sugar-cane</b>	orange	beet	
	<b>pea-bean</b>	grapefruit	
	<b>peanut</b>	<b>egg</b>	

\* Showing the selective abolishment of food sensitivities through sympathectomy in patient A. F. C. The items in heavy type have been eaten without allergic reaction since the operation. These food-allergens are arranged in groups in the order of their recognition as excitants and/or their elimination from the diet.

the sensitivity to some of the major allergens has been abolished, that to some of the weaker ones and even to one of the weakest—milk—persists. Evidently then, the sympathectomy did not result in a nonspecifically increased tolerance to the H-substance, such as can be induced by injections of histamine. The selectivity of the effect of the sympathectomy recalls, rather, the *specificity* of the sensitivities concerned. This astonishing conclusion seems so important practically, as well as theoretically, that I should like to support it with other illustrations.

TABLE 3 shows the unselective tolerance toward the weaker allergens in patient C. T., induced by daily repeated injections of histamine diphosphate:

This was a young woman employed by the Metropolitan Life Insurance Company, where a diagnosis of "nervous and emotional instability—incurable" had been made. Her chief symptoms were severe dizziness, depression, hysteria, abnormal tiredness, "fluttery heart" (probably extra systoles), neuralgia, and constipation. The foods listed in the first column are those first identified as allergens in this case. In the seven months following the elimination of those foods from the diet, the patient was free from her allergic symptoms.

Her symptoms then began to recur and again disappeared on avoidance of the foods listed in the second column (group 2). A few weeks later, her sensitivity to the weaker allergens began to emerge, beginning with cane sugar, and she gradually lost weight. Within a week after the injection of histamine were begun, she found herself able to eat the foods marked by heavy

TABLE 3\*

<i>Group 1 (major)</i>	<i>Group 2 (medium)</i>	<i>Group 3 (minor)</i>
beef wheat orange grapefruit lemon plum	tomato rice rye corn oat coffee onion	sugar-cane potato banana strawberry aluminum

\* Showing the nonselective abolishment of the sensitivities to the entire "minor" group of allergens and to one of the "medium" group in patient C. T. under daily injections of histamine diphosphate. The items in heavy type could be eaten without allergic reaction.

TABLE 4\*

<i>Group 1 (major)</i>	<i>Group 2 (medium)</i>	<i>Group 3 (minor)</i>
<b>beef</b> <b>wheat</b> orange grapefruit lemon plum	tomato rice rye corn oat coffee onion	sugar-cane potato <b>banana</b> strawberry aluminum

\* Showing the selective abolishment of food-sensitivities with sympathectomy in patient C. T. The items in heavy type have been eaten without allergic reaction ever since the operation. Note that the sensitivity to tomato and potato, which had been suppressed by histamine-injections, remained unaffected by sympathectomy.

type without reaction, and she regained her lost weight at the rate of 2½ pounds weekly. Tests of some of the other foods were followed by recurrence of her characteristic allergic symptoms, including dizziness.

This experience, together with the corresponding one in my own case, teaches that the nonspecific antiallergic tolerance obtained by injections of histamine is quantitatively limited to protection against the weaker (minor) food allergens.

TABLE 4 presents again the list of the original food allergens of C. T. The items in heavy type are those that have been eaten since the sympathectomy without any allergic reaction. In this case also, the antiallergic effect of the operation is seen to be selective. Although sensitivity to three of the

major allergens was abolished, that to the much weaker allergen potato remained. Moreover, the sensitivity to three still weaker food allergens that had not been previously detected now emerged: namely, chicken, egg, and tea. To these was added some chemical allergen of the local water supply.

The third case is different from the two just described, inasmuch as the pulse-dietary analysis conducted previous to the sympathectomy failed to disclose a single nonallergenic food. TABLE 5 shows the record for one day

TABLE 5  
C. W. PULSE RECORD PREVIOUS TO SYMPATHECTOMY

<i>Time</i>	<i>Pulse</i>	<i>Diet: symptoms</i>	<i>Time</i>	<i>Pulse</i>	<i>Diet: symptoms</i>
<i>July 25, 1942</i>			<i>July 27, 1942</i>		
B.R.	79	tomato, eggs, pumpernickel, milk	B.R.	72	tooth-paste, grapefruit
B.	93		B.	102	
30'	96		30'	90	
60'	102		60'	93	
90'	100	ham, milk, pumpernickel, tomato, peach	90'	100	beef
L.	90		L.	99	
	93			94	
	96			99	
	96	beans, lamb, tomato, peach, milk. Shaky today		93	potato, "very jumpy"
D.	99		D.	97	
	98			99	
	97			97	
	97			96	
<i>July 26, 1942</i>			<i>July 28, 1942</i>		
B.R.	77	corn flakes, sugar	B.R.	62	very jumpy, grapefruit
B.	100		B.	95	
30'	102		30'	82	
60'	93		60'	90	
90'	86	"shaky"	90'	106	major seizure, unconscious 5 min., milk
L.	90	cornflakes, sugar	Mid	102	
	95		A.M.	102	
	93			114	
	98			77	pineapple
D.	82	milk	Mid	103	
	85		P.M.	110	
	88			103	
	91			112	

on the patient's usual three-meal diet and for three days on a trial diet of selected, usually single, foods. The patient's chief complaint was idiopathic epilepsy (diagnosis confirmed at Rockland State Hospital, New York). Other symptoms, which disappeared after treatment, were abnormal tiredness, constipation, and canker sores. The operation was performed by Dr. Miscall, August 3, 1942. It is important to note that in the succeeding eight months, that is, in the period before the second pulse-dietary diagnosis was carried out, the number of seizures was "about the same as before the sympathectomy." As a therapeutic measure, then, the sympathectomy

was a complete failure, as it has been in so many other reported instances. Now, let us examine its efficiency as an antiallergic measure.

TABLE 6 shows the patient's record, made at the end of the eight-months period. Noteworthy are the generally lower level of the pulse on a large variety of foods; the characteristic constancy of the normal daily maximal pulse rate, 70-71; the reaction to cane sugar with carryover; and the internal evidence of the practical dependability of the patient's pulse counts. So we see that the seizures, continuing throughout the eight months following the

TABLE 6  
C. W. PULSE RECORD AFTER SYMPATHECTOMY

<i>Time</i>	<i>Pulse</i>	<i>Diet: symptoms</i>	<i>Time</i>	<i>Pulse</i>	<i>Diet: symptoms</i>
<i>April 5, 1943</i>			<i>April 7, 1943</i>		
B.R.	56	pineapple, milk	B.R.	67*	tomato, milk
B.	65		B.	79*	
30'	69		30'	69	
60'	64		60'	69	
90'	65	egg, cheese, tomato, milk, prunes	90'	68	lettuce, egg, milk, tomato, cheese, pineapple
L.	62		L.	65	
	68			69	
	64			64	
	66	chicken, cabbage, (lemon), carrot, milk		65	lamb, milk, carrot, peas, honey, apple
D.	64		D.	64	
	70			75*	
	67			68	
	67			69	
<i>April 6, 1943</i>			<i>April 8, 1943</i>		
B.R.	60	tomato	B.R.	60	pineapple, milk
B.	70		B.	64	
	64			66	
	65			68	
	66	egg, tomato, cheese, milk, pineapple, prune		65	tomato, peas, milk, apple
L.	61		L.	63	
	69			65	
	71			66	
	67	grapefruit, sugar		61	lamb, tomato, beans, apple, honey, milk
Eve.	64		D.	64	
	74			68	
	76			71	
	73			69	
	66				

\* Carry-over from cane sugar.

operation, were due to the daily eating of three of her residual allergens: cereals, potato, and cane sugar. Her other residual allergens are fish, dill pickle, and cascara, each of which has caused a *grand mal* seizure.

In the succeeding four years, this young woman, after the usual secretarial course of instruction, has occupied a responsible position in a well-known business establishment. She has occasionally indulged liberally in forbidden sweets on a Friday evening, has regularly experienced the expected seizure on the following morning at home, has cleared her alimentary canal with



laxatives during the day, and has quite recovered in time for her Monday morning appointment. Epilepsy, with her, has become a purely experimental episode.

The antiallergic effect of interruption of sympathetic nerve-chain function has been dramatically illustrated in two cases with the single injection of procaine into a stellate ganglion. The injections were made by Dr. E. A. Rovenstine at Bellevue Hospital.

Patient E. C., a longtime sufferer from a marked destructive conjunctivitis, chronic rhinitis, and atopic dermatitis, had been found by two well-known allergists to be skin-sensitive to numerous inhalant allergens but not to any foods. With the pulse criterion, he was sensitive to so many foods that sympathectomy was advised. Dr. Miscall approved the suggestion of a preliminary procaine block of the stellate ganglion, which was performed October 4, 1946.

The pulse, previous to the block, had ranged as high as 92 on a succession of single foods and was usually above 81 (his normal maximum). Just before the injection, it stood at 84, and one hour later, at which time he took a quart of milk, it was at 83. Thirty minutes thereafter, it was 75, and it did not rise above 81 in the next 48 hours, excepting a little in the evening, probably on account of exposure to tobacco smoke to which he is very sensitive (pulse up to 104 while smoking).

The block broke at 48 hours, the pulse rising suddenly to 88 and remaining above normal until a second block was established. Under the protection of the blocks, it could be determined that at least sixteen foods could be eaten without allergic reaction. With this information, both Dr. Miscall and the patient agreed to the operation. So also did Dr. Conrad Berens, in whose special care the patient had placed himself.

The information obtained with the pulse-dietary technique in the block of the stellate ganglion was useful not alone in permitting a preview of the permanent benefit to be expected from the sympathectomy. The tissue damage involved in the operation causes an irregular acceleration of the pulse that continues for several weeks, in which period, therefore, the interpretation of any changes in the pulse following a test meal might be undependable. This difficulty was marked and prolonged in the case of E. C., but he could evade it by simply limiting his diet to the sixteen foods (an ample diet) that had been found safe in the period of the blocks.

Patient B. C., for many years a sufferer from atopic eczema with an almost intolerable itching, had been referred to me by Dr. M. B. Sulzberger. With the pulse criterion, she was found sensitive to egg (vomiting and pulse of 90) and to the zinc ointment that she was using (pulse up to 96 in two tests before breakfast). Otherwise, no other allergens could be identified. The pulse constantly ranged above her normal maximum (80).

The injection of procaine (Rovenstine) was made at 12:20 P.M. There was a brief dizziness, and at 12:25 the pulse was 60, rising at 12:35 to 64. The itching ceased immediately after the injection and the patient's usual tenseness was replaced by a complete relaxation. In the succeeding 96 hours, the pulse remained between 64 and 80, excepting after a test with

lamb (88) and grapefruit (88). Both of these tests were followed by a brief recurrence of the itching. Under the protection of the block, 18 foods could be identified as nonallergenic.

### *Discussion*

As we have seen, the pulse-dietary technique provides a dependable objective measure of the antiallergic effect of sympathectic ganglionectomy or procaine block. It must be emphasized that, in my joint study with Miscal, Rovenstine, and others, these procedures have been employed only in conditions that have been proved, through the pulse-dietary method alone, to be allergic. These have been hypertension, epilepsy, vertigo, psychoneurosis, gastrointestinal allergy, stammering with tic, migraine, irritability, destructive conjunctivitis, allergic eczema, and urticaria. Moreover, they have not been applied in the cases that have been easily solvable with the dietary regime but in those difficult cases that are found sensitive to too many important foods. In our experience, then, the operation, properly employed, supplements the dietary procedure, increasing the effectiveness of the latter to a high percentage of clinical success, depending somewhat on the intelligence, character, and economic circumstances of the patients.

It has not yet been known to abolish sensitivity to an inhaled allergen (dust, perfume, tobacco, cement, paint fumes, *etc.*).

Sympathectomy offers a smaller prospect of usefulness in subjects sensitive to only few foods, since the few sensitivities *could* be just those which would by chance not be abolished by the operation in the particular person. However, the "prognostic stellate block" can be profitably employed even in such cases, and the decision as to operation can be made according to the outcome.

Finally, it should be emphasized that, according to the results of my study of the antiallergic effect of sympathectomy, the operation should no longer be considered as a therapeutic measure for the relief of particular symptoms but almost always as a means of lessening the extent and severity of the food allergy that causes them and so facilitating its complete dietary control.

### *A Brief Critical Review of the Literature Concerning Sympathectomy and Sympathectic Procaine-Block*

Having set forth the details of my own special use of these procedures, I can more readily discuss their different use by earlier experimenters.

Sympathectomy was employed as a therapeutic measure from the latter part of the 19th Century. It was applied locally for the relief of many symptoms, which at the time were not (and indeed still are not) generally believed to be due to the same cause (certainly not an allergic one, although some were conceived to be caused by some mysterious neurogenic "imbalance"). The list includes Raynaud's disease, thromboangiitis obliterans, asthma, cardiospasm, constipation, migraine, paroxysmal tachycardia, neuralgia, angina pectoris, essential hypertension, chronic arthritis, and epilepsy. Most of these have now been shown to be allergic, that is, they represent variously localized manifestations of a single, hereditary, constitutional disability.

The earlier experimenters proceeded according to their hypothesis that the symptoms were due to some local neurogenic abnormality ("imbalance of sympathetic and parasympathetic control") and thus to the logic that the gangliectomy should be done close to the supposed source of the symptoms and with meticulous thoroughness. Thus, for migraine and facial neuralgia, the cervicodorsal ganglia are removed and the vertebral and carotid arteries may be stripped. For essential hypertension of supposedly renal origin, on the other hand, the favored operation is now removal of the upper lumbar ganglia and both celiac ganglia and even more extensive destruction.

I have called attention elsewhere<sup>1</sup> to the consensus of surgical observers that removal of lumbar and celiac ganglia was frequently followed by relief of headache and other symptoms, even when the hypertension persisted. This "paradox," as it is called by some operators, seems not to have disturbed the general assumption of a local action of sympathectomy. Unfortunately, also, the stony conservatism of some neurosurgeons has so blinded them to the clear significance of those reports that they still refuse the benefit of the conservative operation that I have described, even to the most desperate case of migraine.

We reach the conclusion, then, that the essential benefit of sympathectomy derives from its constitutional antiallergic effect and that the selection of the site of its performance should be determined not according to the locality of the chief symptom but according to general surgical principles.

In one case (E. C.) a left cervical ganglionectomy was done. Shortly thereafter, there was improvement in vision and in the conjunctivitis, but this was limited to the right eye. There was also a general clearing of the atopic dermatitis, but the left side of the face and left ear, the side of the operation, remained unimproved. This one experience is not conclusive, but it suggests a possible *unfavorable* local action of the operation in some instances.

For the present, the procedure of choice would seem to be the removal of two or three lumbar ganglia on one side only, wherever the chief food-allergic symptom may be located.

The ganglion block that has been used by Rovenstine as a valuable preliminary aid in the application of the antiallergic sympathectomy has been used for a similar purpose since 1925 by Mandel,<sup>2</sup> Swetlow,<sup>3</sup> White,<sup>4</sup> and Flothow.<sup>5</sup> At first, alcohol was recommended and, later, this was preceded by procaine. In a single experiment, we found that the injection of alcohol (at the second block in patient E. C.) caused a persistent moderate tachycardia which interfered with the interpretation of the pulse-dietary record.

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STRUCTURE IN RELATION TO CELLULAR  
FUNCTION\*

*Conference Chairman:* ROBERT CHAMBERS

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# THE CELL AS AN INTEGRATED FUNCTIONAL BODY

By Robert Chambers

*Department of Biology, New York University, New York, N. Y.*

The title of this monograph refers to a subject which has been a problem for consideration ever since the protoplasmic body was realized to be the physical basis of the life-giving properties of a cell. It was surmised early that protoplasm with its varied activities could not be a homogeneous substance, hence the several hypotheses of structure with which we are all familiar. It was not, however, until within recent years, when chemistry has so rapidly evolved into a science of structure, that we have been able to introduce logic into our investigations on the relation of structure to protoplasmic function.

Our endeavor here is to touch on characteristics which are common to cells in general. For this reason, we have avoided certain structures of highly specialized cells, such as of muscle and nerve. The subjects treated may well serve as introductory to separate symposia where each can be discussed in greater detail than is possible here.

An adequate study of protoplasm requires an understanding of many facets. These may best be investigated separately in cells in which certain features are intensified—for example, that of reversible contraction in muscle, of conduction in nerve, of solation and gelation in ameboid cells, *etc.* It will then be necessary to combine all available studies to gain insight into the properties of the protoplasmic body common to all cells.

These remarks are intended to present a general morphological concept of a cell as a whole. More particularly, I shall deal with that part which we are terming its extraneous coat in contrast to the surface film of the enclosed protoplasmic body. Cytolysis is involved only when the protoplasmic surface film is broken. Within the protoplasm are regional differentiations which must be considered as intimately related to protoplasmic structure as a whole. Finally, we come to cell division, a property common to plants and animals, an understanding of which process requires examination of their similarities.

It is well known that the earliest cell to be recognized was the plant cell and that the original definition was that a cell consists of a wall surrounding a cavity. We might do well to return to this with the addition introduced by von Mohl, that the cavity contains a body of protoplasm. Purkinje confused the issue by referring to the animal cell as being synonymous with protoplasm. It was not till Curt Herbst presented evidence for an enveloping material, by means of which neighboring cells are stuck together and which can be dissipated in Ca-free media, that we were able to bring into harmony the original concept with that of the present. Normally, the protoplasm of a cell is not naked, but is covered with extraneous coats of varying rigidity. It is these coats which we regard as supporting walls. In animal cells, there is evidence that these coatings are the sloughed-off by-products of the protoplasmic surface. They can be experimentally

removed without sacrificing the life of the protoplasmic body. In plant cells, the extraneous coats of the animal cell are represented by the so-called middle *lamellae*, which lie between the cellulose of neighboring cells or cover the surfaces of single cells. The middle *lamella* consists of salts of polysaccharide pectates. In common with the extraneous coats or walls of animal cells, the pectate wall of plant cells also requires calcium for its structural rigidity. The normal sticky consistency of these external coats depends upon a fairly precise balance in the proportional concentrations of Na and of Ca in the medium, the proportion which exists in Ringer's solution. A decrease in the calcium content or an increase in the acidity of the medium tends to soften and to disperse the pectate *lamellae* of the plant cell and the protein-like extraneous coats of the animal cell.

In studies on permeability, the presence of this material is a factor to be considered, especially when one deals with sheets of coherent cells. Calcium in the medium stiffens and tightens up the intercellular cement so that the resulting decrease in porosity of a cellular membrane is to be explained not so much from changes in the cells themselves as from changes in the cement between the walls. A striking example is the porosity of the blood-capillary wall, which is bathed with a fluid consisting of a balanced salt solution containing calcium. The freedom of the calcium ion to form combinations with the cement varies with physiological changes in pH of the medium.<sup>1</sup> Moreover, changes in the tonic state of the individual endothelial cells may cause the cells either to spread and thin out or to draw in and thicken, thereby narrowing or widening the spaces occupied by the cement between them. The consequent variations in the packing or dispersing of the cement appears to be Nature's way of causing the blood-capillary wall to maintain a readily shiftable state of porosity without affecting the permeability of the constituent cells of the endothelium.

Furthermore, when we deal with single cells, the investigations, for example, on the effect of electrolytes on their water permeability, must take into consideration the possibility of the mechanical effect of a weakened or of a stiffened extraneous coat in permitting or preventing the swelling of cells by the water intake. The stiffening action of calcium on the extraneous coats of sea-urchin eggs might be sufficient to explain the findings of Lucké and McCutcheon<sup>2</sup> that calcium salts decrease the permeability of the eggs to water.

It is of great importance that we realize the existence of such extraneous coats. Jacques Loeb's conception regarding the antagonistic action of salts for the preservation of life may be due, in large part, to the preservation of the extraneous coats of cells by a definite proportion of sodium and of calcium in the medium. The proportional concentration of NaCl and of CaCl<sub>2</sub> which R. S. Lillie<sup>3</sup> found to be necessary for preserving the "jelly" which surrounds many echinoderm eggs is the same as that required for extraneous coats in general.

In this discussion on the existence of extraneous coats or cell walls, I am not referring only to material which may be adsorbed, such as the proteins existing in body fluids and in blood, but mainly to coatings which arise

from the cell protoplasm as by-products and are sloughed off from it to form a coat in the presence of calcium or to dissipate in its absence.

A significant experiment which shows the difference in the action of Ca on the extraneous coats and on the naked protoplasmic surface is the following: Unfertilized sea-urchin eggs were immersed in a solution of 0.4 M  $\text{CaCl}_2$ , a concentration which is isotonic with sea water. The extraneous coats of these eggs quickly harden and the eggs behave as rigid balls. When a drop of the  $\text{CaCl}_2$  solution containing the eggs is deposited on a coverslip, the eggs stick tenaciously to the glass surface. The stiffened coat can be broken and torn with microneedles. The application of pressure with a microneedle on the cracked shell will expel the naked protoplasmic body of the egg, much as a pea can be shelled. The extruded, naked egg immediately assumes the shape of a sphere, it is non-sticky, it can be rolled about and can be pinched into several pieces which immediately round up, each spherule retaining the appearance of normal egg cytoplasm. The spheres behave like a droplet of oil with a flowing, liquid surface. The pinching has to be done with the cylindrical side of the glass microneedle, and the performance can be repeated as long as the surface of the protoplasmic body is not scraped with a sharp edge or the pointed tip of the needle. When this is done, the tear opens and the entire body of the sphere is quickly converted into a mass of frothy, coagulated material. The naked egg spheres can be capped with a drop of oil<sup>4</sup> of appropriate interfacial tension. The liquid state of the surface film enveloping these spheres is demonstrated by the ease with which the capped oil can be moved from place to place and be divided into two separate caps on the surface of the sphere. The effect closely resembles that of a drop of paraffin oil lying on the surface of water, where the oil can be shifted in any direction on the surface but cannot be pulled off.

Evidently, when calcium is present in the medium, the extraneous coats are stiffened, while the protoplasmic surface film is not.

When eggs are denuded of their extraneous coats by shaking and washing in a Ca-free medium and then returned to calcium-containing sea water, the phenomenon of oil-capping also occurs, but in this case the oil cannot be moved about. It would appear that extraneous coat material continues to develop in sea water and forms a covering over the surface of the egg in sufficient quantity to prevent the oil cap from being shifted.

The protoplasmic surface film presumably consists of a palisade-like structure of lipo-protein complexes so arranged that the protein part of the complex is directed to the exterior where it is in direct contact with the surrounding sea water. In sea water, the protein portion of the complex may continually be sloughing off and be denatured to constitute the extraneous coat. It would seem that this does not occur appreciably when the surrounding medium is purely  $\text{CaCl}_2$ .

The mobile protoplasmic surface film can be retained in the total absence of calcium in the medium, such as in an isotonic solution of citrated NaCl and KCl. There is evidence, however, that the sloughing off of protein-like material continues to occur in such a solution but that, in the absence



of calcium, the material becomes dispersed so that no appreciable extraneous coat develops.

We regard the protoplasmic surface film or plasma membrane as a structure which is constantly forming and being renewed. This structure, as long as it is a continuous envelope, maintains the integrity of the protoplasm within and the cell remains alive. The protoplasmic surface film cannot be removed or torn without causing damage. A quick repair of the film may result in recovery. Otherwise, the damage becomes irreparable, whereupon the remainder of protoplasmic surface film dissipates and the exposed interior undergoes disintegration.

We now come to the problem of what is entailed in cytolysis. A protoplasmic body is highly plastic. It can be greatly compressed and distorted. It can be churned<sup>4</sup> to the degree that all parts, including its surface, are displaced without undergoing cytolysis. It is only when the surface film is torn open that cytolysis ensues. The cytolysis can be localized only if the gap of the tear is quickly repaired by the rapid formation of a protoplasmic film beneath the cytolized region. In media containing calcium, the cytolytic effect is a conversion of the protoplasm into a dead, sticky, and often frothy coagulum. In the absence of calcium, this does not occur. Instead, the gap produced by the tear opens up, whereupon the granular contents pour out and become dissipated in the calcium-free medium. Under these conditions, the question arises to what extent the integrated constituents of the protoplasm persist so that the granules which are seen to scatter constitute the vital constituents of the protoplasm, or whether other granules appear as products of the cytolytic breakdown. Much work is needed to elucidate this problem.

I wish, here, to refer briefly to the physical state of the protoplasm beneath its surface film. There seems to be no question about the existence of a firm, gelated cortex of appreciable thickness immediately underlying the more mobile, fluid, protoplasmic surface film. Under certain circumstances, the film can be elevated off the cortex. An example of such an occurrence is the formation of the delicate cone-like protrusions on an egg at the site of sperm entry.<sup>4</sup>

The gelated state may occupy the entire interior of the cell. Even in such highly fluid eggs as the *Arbacia* ovum there is evidence that the interior is not a true sol, but a weak, tenuous gel. Apparently, spontaneous variations occur, so that considerable precaution is required in viscosity studies as tested by centrifugation or by the exhibition of Brownian movement.

A similar conclusion, that the cytoplasm of the ameba is normally in a state of incipient gelation, was suggested by Harvey and Marsland<sup>6</sup> in their studies on the ameba viewed through the centrifuge microscope. They noted that the larger crystals in the cytoplasm fell in "jerks" as they were driven by the centrifugal force through a visibly clear field. Moreover, this may have been due not so much to sudden breaks in a colloidal mesh in the fluid cytoplasm, but more likely to occasional sticking of the crystals to the inner border of the peripheral plasma gel. A worth-while check might be to examine the behavior of the crystals in an ameba, the ecto-

plasmic gel of which has been experimentally solated prior to its being exposed to the action of the centrifuge microscope.

In any event, one of the most obvious properties of protoplasm is that of reversible gelation and solation. A gel, no matter how tenuous, offers a consistent structure with plenty of surfaces for the manifold adsorptive and enzymic processes on which protoplasmic function depends.

Concerning the problem regarding the ultrastructure of protoplasm, I wish to refer to the very ingenious conclusion drawn some years ago by A. R. Moore.<sup>6</sup> It is well known that the ameba-like plasmodia of *Myxomycetes* creep through fine pored meshes and reconstitute themselves by fusing together on the other side of the mesh. Moore found that the plasmodia could also crawl through large molecule-sized porcelain filters. However, he observed that viable plasmodial masses appeared on the other side of the filter only when the plasmodium was allowed to crawl through of its own momentum. If he exerted pressure to drive it through, there resulted a breakdown of the protoplasm and cytolysis. He interpreted his experiment to mean that the protoplasm consists of long, fiber-like molecules which, when allowed to orient themselves properly, passed readily through the pores of the filter. However, when extraneous force is applied, many of the fibers would be presented crosswise to the pores and break instead of slipping through. He assumed that this initiated the cytolysis. Here again, there is need of caution. Undue pressure or crushing breaks the protoplasmic surface film which envelopes the creeping plasmodium. This alone, by exposing the interior to the environment, may suffice to induce cytolysis.

The relative inertness of the proteins in the living cell is an interesting feature. This has been suggested by the consistency with which solutions of color indicators of overlapping pH values, when injected into living cells, give color virages pointing to the same pH value of approximately 6.8.<sup>7</sup> It would appear that the proteins present are not sufficiently active to introduce any appreciable protein error such as obtains in the test tube. A similar conclusion based on more positive evidence has been arrived at by Dr. Kopac, which he discusses in a subsequent paper in this monograph.

The fact that there is sufficient ionic activity in the continuous aqueous moiety of protoplasm is indicated by the existence of an appreciable buffering capacity of the protoplasm. A feature of this is the ability of an ameba to tolerate the injection of picric acid. The tolerated amount could be determined by injecting a mixture of picric acid and brom cresol purple. If the injected fluid retains its yellow color, the acidity of the fluid has overcome the protoplasmic buffers and the ameba quickly succumbs. On the other hand, a change of the color to green indicates that the brom cresol purple has assumed the blue color of its alkaline range by the ability of the protoplasm to buffer the introduced acid. These green-colored amebae survive and move about apparently quite unaffected.

The existence of a physiological relationship between the nucleus and cytoplasm is generally accepted. Ocular evidence of such a relationship has been accumulating in the literature, such as the recovery of a fibroblast

from the destructive effect of puncturing the nucleus provided a second nucleus is present in the cell<sup>8</sup> and the experiments which indicate a high degree of porosity of the nuclear membrane of the ameba.<sup>9</sup> Further evidence is presented by Dr. Duryee in this monograph. I wish to emphasize the point that there are certain periods in the life history of a cell during which the interrelationship is carried to the extreme of an actual commingling of the nucleoplasm with the cytoplasm. This occurs prior to proliferative activity of the cell, as in mitosis and strikingly during maturation of an ovum. The commingling of the nuclear sap of the germinal vesicle with the cytoplasm results in the conversion of the cytoplasm into nucleocytoplasm, every part of which is now capable of being induced to undergo cleavage. It is this faculty which in all probability enables a mature ovum to undergo repeated cleavages over a relatively short period of time.

I conclude with a description which indicates the close similarity between plants and animals in their method of cell division. You are aware that the conventional description for an animal cell is that it divides by constriction at the equator, while the plant cell divides by the deposition of a wall across the equator with no evidence of constriction. Actually, we should regard the separation of the two daughter cells as the end stage and only incidental to an earlier and more fundamental process of separation of the protoplasmic bodies of the cell. In this we come back to my thesis at the beginning of my remarks that we must distinguish between the cell and the enclosed protoplasmic body.

Let us consider an animal cell, either the spermatocyte of a grasshopper<sup>10</sup> or the ovum of a sea urchin. The metaphase of the mitotic spindle forms; then the separation of the chromosomes occurs as the anaphase advances into the telophase and the two daughter nuclei begin to be reconstituted. During this period, the animal cell undergoes a certain degree of lengthening (karyokinetic lengthening, first described by Oscar Hertwig), but still with no sign of constriction at the equator. Now a phenomenon occurs which is well recognized in plant but not in animal cells. The so-called telophasic spindle-remnant, at the equator, where the chromosomes had been during metaphase, spreads equatorially, pushing ahead of it all cytoplasmic components which lie in its way until the spreading margin of the spindle-remnant reaches the equatorial periphery of the cell. The separation is now complete between the protoplasmic bodies of the two incipient daughter cells. Not until this is completed do we have the onset of the final phase, which in the plant cells is the deposition of an intervening wall, and in the animal cell an inward advance of the walls of the division furrow.

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## SURFACE PROPERTIES OF THE ERYTHROCYTE

By M. H. Jacobs

*Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania*

The surface of the mammalian erythrocyte has been more studied, by a greater variety of methods, and on the whole with greater success than that of any other cell. There are two chief reasons why it has received so much attention. The first is that it can be physically separated from the cell contents in a manner that is unique among cells. The second is that suspensions of erythrocytes have certain optical properties that are peculiarly favorable for exact experimental work.

Both peculiarities of the erythrocyte are connected with its high degree of specialization for gas transport in the body. In it, internal structures have been reduced to a minimum, and one internal constituent, hemoglobin, has been increased to a maximum. In hemolysis, the hemoglobin escapes from the cell, and little is left but a ghost-like and doubtless somewhat modified cell surface. Erythrocyte "ghosts," suitably purified and concentrated, have been extensively studied by chemical, optical, electrical, and immunological methods.

Furthermore, when the erythrocyte loses its hemoglobin, it becomes almost completely transparent. This loss can be brought about by various alterations of its surface, and special optical methods which are both simple and accurate are, therefore, available for the investigation of surface changes that lead to hemolysis. Similar methods can also be used to study the non-hemolytic volume changes associated with the passage of solutes in either direction through the cell surface. Not the least of the advantages of the erythrocyte for such studies is the fact that the results obtained with it are usually statistical beyond the wildest dreams of the biometrician. Only rarely does an experiment on the erythrocyte involve the use of less than a quarter of a billion of individual cells, *i.e.*, the number contained in one drop of human blood.

By the isolation and study of erythrocyte ghosts, important information has been obtained about the general characteristics of its surface. Since I have done little work of my own in this field, I shall merely summarize very briefly some of the more important conclusions arrived at by other workers.

The erythrocyte ghost has the characteristics of a non-liquid membrane, of the order of perhaps  $100 \text{ \AA}$ ,<sup>1, 2</sup> or a little more, in thickness. It contains most of the cell lipids,<sup>3</sup> approximately sufficient in quantity to form a bi-molecular layer,<sup>4</sup> and large amounts of protein of a rather peculiar nature,<sup>5, 6</sup> together with blood-group substances<sup>7</sup> and other chemical materials. It has a high electrical resistance and a large electrical capacity.<sup>8</sup> Dr. Waugh, in the following paper, will discuss its very interesting optical properties. For our present purposes, it is sufficient to note that according to Schmitt and his co-workers<sup>9, 10</sup> it behaves as if it contained radially arranged lipid-like molecules and tangentially arranged protein elements.

Various hypothetical models have been proposed to account for these and other known properties of the erythrocyte surface. The simplest of these may be chosen as a basis for discussion, with no present expression of opinion as to its adequacy. In it, the non-polar ends of two layers of radially arranged lipid molecules adjoin each other. The opposite, more polar ends adjoin and may even be in chemical combination with an inner and an outer layer of proteins. Such a model, according to Danielli,<sup>11</sup> while metastable rather than stable, is at least much less unstable than several other conceivable arrangements of protein and lipid molecules.

My own views as to the nature of the surface of the erythrocyte have been derived chiefly from studies of its permeability to various solutes. The remainder of the space at my disposal will be devoted to some of the inferences that can be drawn from such studies. Six principles will be mentioned which seem to govern the behavior of the erythrocyte toward dissolved substances. Any satisfactory hypothetical picture of its surface at best should explain all these principles and at worst should not be incompatible with any of them.

#### *Principle 1. Non-Specific Solubility*

About fifty years ago, Overton<sup>12, 13</sup> formulated the principle that the ease of entrance of many substances into living cells is closely paralleled by their solubility in fats and fat solvents or, more accurately, by their partition coefficients between these substances and water. He therefore postulated the presence at the surface of the cell of a layer of material having the general physical properties of a fat-like substance.

Somewhat differently expressed, Overton's principle seems to mean that non-polar hydrocarbon groupings in organic molecules have a general affinity for solvents in which a non-polar hydrocarbon structure predominates. Other things being equal, the higher this affinity in the case of a given solute the greater its partition coefficient between an organic solvent and water, and the more rapid, as a rule, its entrance into living cells, regardless of its molecular volume. On the other hand, polar groups with an affinity for water have the opposite effect, with respect both to partition and to cell permeability. The latter effect is seen with especial clearness in the ionization of many weak organic acids and bases.

Erythrocytes, like nearly all other cells, show many instances of this non-specific solvent-like behavior. Beef erythrocytes, for example, are among the least permeable mammalian erythrocytes to glycerol (molecular weight, 92). Something like three quarters of an hour is required for enough glycerol to enter them from a solution isosmotic with blood to bring about their osmotic hemolysis. But the acetic acid ester of glycerol, monoacetin (molecular weight, 135), despite its higher molecular volume, enters them to the same extent in perhaps 40 seconds. The still larger but more lipid-soluble molecule of diacetin (molecular weight 178) does so in possibly 6 seconds.

The same principle is illustrated by a homologous series of, for example, saturated fatty acids.<sup>14, 15</sup> In the series, acetic, propionic, butyric, and

valeric acids, which differ only slightly in their strengths as acids and are, therefore, particularly favorable for such comparisons, the rate of entrance into the erythrocyte increases with increasing lipid solubility despite increasing molecular weight, in the manner demanded by the principle of Overton. Many other similar examples could be given.

A further likeness in the behavior of the erythrocyte, of many other cells, and of non-specific organic solvents is found in the effect of pH on the uptake of organic acids and bases from an aqueous solution. In general, the uptake of a weak base such as an alkaloid by either an erythrocyte or an organic solvent is favored by alkalinity and hindered by acidity. That of a relatively weak organic acid such as salicylic acid is favored by acidity and hindered by alkalinity.

It seems difficult to account for this universal non-specific type of entrance of even very large molecules into cells without postulating some rather extensive portion of the cell surface in which they can, so to speak, dissolve. Most of the cell lipids are present in the isolated erythrocyte ghost so that a bimolecular layer of them would obviously serve very admirably as a non-specific solvent, though this particular arrangement is by no means the only possible one.

As for the proteins of the cell surface, their hypothetical locus must clearly not be such as to interfere with the free passage of even very large lipid-soluble molecules. An arrangement of the lipids and proteins in parallel, as in a mosaic, would, of course, meet the needs of the situation. An arrangement in series, however, would also be compatible with the observed behavior of these particular molecules if the groupings of the proteins were sufficiently loose to give ready access to the underlying lipid layer. As a matter of fact, there is evidence of another sort that, although proteins are probably present at the surface of the erythrocyte, they by no means form a barrier to non-polar molecules. This is the observation of Mudd and Mudd<sup>16</sup> that erythrocytes introduced into an oil-water interface, unlike leucocytes, go into the oil. The presence of additional substances at the surfaces of sensitized erythrocytes, however, may destroy their affinity for oil.<sup>17</sup>

### *Principle 2. The Molecular Sieve Mechanism*

The erythrocyte, like other cells and certain artificial membranes, is, in general, more permeable to very small molecules than to those of larger size. Indeed, molecules which are sufficiently small seem to enter it with great ease, however low their affinity for lipids.

As a molecular sieve, the surface of the erythrocyte behaves as if it were either very thin or very porous, or both. Its permeability to the most abundant of all small molecules, water, is greater than that of any other known cell. Lucké<sup>18</sup> has tabulated comparable permeability constants for a considerable number of cells of both plant and animal origin. Expressed in the same units, they range from about 0.1 to 1.0. The corresponding figure for the erythrocyte is about 3.0,<sup>19</sup> that is, 3 to 30 times as great. Even this figure is probably too low, since it depends upon a

hemolysis method, and the escape of hemoglobin from the cell is by no means instantaneous. For a time, the endothelial cells of the capillary wall<sup>20</sup> were thought to be even more permeable to water than the erythrocyte, but, according to Zweifach,<sup>21</sup> it is the permeability of the intercellular substance rather than that of the cells themselves that is here involved.

The permeability of the erythrocyte to small hydrophilic molecules varies so greatly from species to species that generalizations are difficult. At the one extreme, beef erythrocytes<sup>22</sup> show a slightly lower permeability to ethylene glycol and glycerol than do cells of the plant, *Chara*,<sup>23</sup> and *Arbacia* eggs.<sup>24</sup> At the other extreme, the erythrocytes of some rodents, particularly the groundhog,<sup>25</sup> show evidence of a porosity greater than that of any other known animal cell (provided that the observations of Zweifach, mentioned previously, permit the exclusion of the cells of the capillary endothelium).

Further light on this type of permeability seems to be thrown by a study of the effect on it of different temperatures. Temperature coefficients ( $Q_{10}$ ) for the rates of entrance of different hydrophilic solutes vary widely, *i.e.*, from about 1.4, which is typical of a simple diffusion process, to 6.0 or more, but with a very general tendency of the values, with the same cell, to increase with the molecular weight of the penetrating substance.<sup>26</sup> High temperature coefficients are frequently supposed to indicate that some chemical reaction is involved, but there is no independent evidence to suggest that this is true of the entrance of most solutes into the erythrocyte. Even if it were, there is no obvious reason why the thermal characteristics of the reactions in question should be so directly related to the sizes of the diffusing molecules. Much more plausible is the suggestion of Danielli and Davson<sup>27</sup> that the diffusing molecules must force their way through a barrier of some sort, and that only those individual molecules succeed in doing so whose kinetic energy reaches a certain critical value.

What part of the cell surface is capable of acting as a molecular sieve? Collander<sup>28</sup> has studied artificial protein membranes and found them to have sieve-like properties. But a uniform protein layer at the surface of the erythrocyte, in series with the lipids, could hardly act in this way. If its meshes were large enough to provide a ready passage for large lipophilic molecules, it could hardly exercise a selective action according to size on much smaller hydrophilic ones. It would seem that an effective sieve-like protein structure would have to be arranged in parallel with the lipids—which is essentially the old mosaic theory of cell permeability. It is entirely possible, however, that the lipids themselves might act as the molecular sieve. Rideal<sup>29</sup> and Langmuir<sup>30</sup> have shown that a monolayer of a fatty acid permits a fairly ready passage of water molecules through it. The more complex phospholipids of the erythrocyte might easily provide a still greater degree of porosity.

### *Principle 3. Species Differences in Permeability*

In their permeability to hydrophilic solutes, specific differences among erythrocytes are both striking and numerous. First, as has just been mentioned, there are differences in the apparent pore size of the molecular



sieve. The erythrocytes of the beef and sheep admit the 3-carbon glycerol molecule with some difficulty. Those of the groundhog are entered readily by 4-, 5-, and even 6-carbon compounds.

Of greater interest are cases in which differences in chemical structure rather than in mere molecular size are involved. For example, human erythrocytes are very slightly permeable to the 4-carbon polyhydric alcohol, erythritol, but they are entered with considerable ease by the 5-carbon sugar, xylose, and they even have a permeability, not found in most other species, to the 6-carbon sugar, glucose. In the mouse, conditions are the exact reverse. Its erythrocytes show little permeability to any of the sugars, but they are unusually permeable to the 4- and 6-carbon polyhydric alcohols, erythritol and mannitol.

The molecules of mannitol and glucose are so large that any pores in a simple molecular sieve which would admit them should also admit the smaller molecules of erythritol and xylose. A door that the cat could enter would not keep out the kittens. Some factor other than molecular volume or lipid solubility—the latter being very low for all the substances mentioned—seems to be operating in such cases.

Specificity is not confined to large molecules, but extends to those of small size as well. Urea and ethylene glycol both have low and approximately equal molecular volumes. But, whereas most cells, including the erythrocytes of all the lower vertebrates, are more permeable to ethylene glycol and sometimes even to glycerol than to urea, mammalian erythrocytes are incomparably more permeable, *i.e.*, 100 times or more, to urea than to ethylene glycol, or indeed to most other substances.

Glycerol penetrates some erythrocytes (*e.g.*, those of man and the rat) at a rate that is much faster than would be predicted from its molecular volume and low lipid solubility. It also seems to enter the erythrocytes of most of the birds that have so far been studied as fast as, or sometimes even faster than its smaller homologue, ethylene glycol. The erythrocytes of the reptiles, on the other hand, show a very low permeability to glycerol.

Among the mammals, the erythrocytes of almost all the carnivores so far studied show a peculiar sensitivity to the action of alkalinity that is not found in other groups. Even very closely related species of mammals may show characteristic differences in permeability. We have found at least a dozen apparently constant differences in permeability between the erythrocytes of the albino rat and the albino mouse.<sup>31</sup> For my own amusement, rather than for any useful purpose, I have constructed an analytical key, based on permeability characters and similar to those used by systematic zoologists and botanists, which permits the identification of the erythrocytes of some 20 species of mammals.

This remarkable specificity of the cell surface has not been observed to apply to lipid-soluble substances, which show a great similarity of behavior with all erythrocytes and with most other plant and animal cells as well. In the case of the highly varied behavior of the hydrophilic substances, however, it seems impossible to account for the known facts, of which only a few have been mentioned, without introducing into our hypothetical

picture of the cell surface a rather high degree of chemical complexity, involving the presence of substances other than lipids and proteins. What the nature of these substances may be, and how they are arranged in the cell surface, is still a matter for speculation. But increasing knowledge is not likely to simplify any picture that has so far been suggested.

#### *Principle 4. Special Permeability to Anions*

A special permeability to anions seems to be peculiar to the erythrocyte and is known to play an important part in the transport of carbon dioxide by the blood. This permeability is sufficiently great to permit large exchanges of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  ions during the three quarters of a second or less which the blood spends in passing through the capillaries of the lungs, with a reversal of the exchange during its passage through the body tissues. A similar rapidity of exchange has been shown by several methods to occur *in vitro*, and is known to be possible in the case of many other relatively small anions such as  $\text{OH}^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , etc. The erythrocyte, however, is practically impermeable to large anions such as ferricyanide, citrate, tartrate, etc.

With regard to cations, the situation is somewhat complex. It is certain that a free permeability to them cannot exist. If it did, the erythrocyte could not maintain its integrity, since it is easy to show that simultaneous osmotic and ionic equilibria could not exist at a finite cell volume. On the other hand, there are observations which indicate that the impermeability of the erythrocyte to cations is neither purely physical in nature nor entirely complete. According to Harris,<sup>32</sup> metabolic processes in the erythrocyte are involved in the retention by it of potassium. Its impermeability to this ion therefore, at least in part, may be a physiological one. Experiments with radioactive potassium and sodium<sup>33, 34, 35</sup> also show that slow passages of these ions across the erythrocyte surface can occur, but that the times required are measured in hours rather than in fractions of a second, as is the case with anions. Practically speaking, therefore, the older concept of the erythrocyte as a cell freely permeable to anions and impermeable to cations is still valid for the interpretation of brief experiments, though more complex metabolic factors must be taken into account when the times are long.

There is good reason to believe that the differential permeability of the erythrocyte to ions is associated with the protein portions of its surface. One piece of evidence is the fact that this differential permeability is destroyed by a variety of agents that denature proteins. It has been suggested, from time to time in the past, that many kinds of hemolysis may fundamentally be due to a conversion of a cation impermeability into a cation permeability by the hemolytic agent. This view has recently been elaborated and given experimental support by Wilbrandt,<sup>36</sup> who has proposed the term "colloid-osmotic hemolysis" for the indirect osmotic hemolysis resulting from cation permeability.

One agent which produces changes of this sort in an easily controllable way is butyl alcohol<sup>37</sup> in concentrations approaching saturation of an aqueous

solution. The effect falls off rapidly with decreasing concentrations. It is very easy to demonstrate the production of cation permeability with butyl alcohol. The procedure is to expose the cells for an appropriate time to a high concentration of the alcohol in an isotonic solution of sodium chloride or of sucrose and, then, before hemolysis has occurred, to dilute the mixture with a considerable excess of isotonic sucrose, which reduces the concentration of the alcohol to a point at which it has little further effect on the cells. If the exposure of the cells is too long, they become permeable to sucrose as well as to sodium. If it has been properly chosen, however, a suspension is obtained of erythrocytes which are stable in sucrose, but which almost instantly undergo hemolysis when placed in a salt solution. Their volume changes, in different mixtures of sucrose and salts and in salt solutions of different pH values, are in good agreement with the theoretical predictions for cells permeable to both anions and cations. Other methods of denaturing the proteins may also be used to obtain cation-permeable cells—for example, by heating the cells, either with or without the addition of a substance which favors protein denaturation, such as salicylate or urea, and then quickly cooling them in a solution consisting chiefly of isotonic sucrose.

What is the location in the cell surface of the proteins responsible for differential ionic permeability? If the arrangement were in series with the lipids, it is difficult to imagine that any superficial wide-meshed layer of protein through which large lipid-soluble molecules could readily pass would delay for hours the passage of, for example, radioactive potassium ions. Apart from this difficulty, however, would be that of accounting for the passage of hydrophilic ions through the strongly hydrophobic underlying lipid layer. Indeed, the high electric capacity of cell suspensions, including those of erythrocytes, is generally interpreted as indicating extensive ion-impermeable regions, which are presumably furnished by lipids.

It seems plausible, therefore, to suppose that any protein regions which show differential permeability to ions are in parallel with the lipids rather than in series with them. This is essentially the conclusion arrived at by Cole and Curtis as a result of electrical studies on cells of a very different nature. With the giant axone of the squid<sup>38</sup> and with *Nilella*<sup>39</sup> cells, they found on stimulation an enormous increase in electrical conductance, indicating increased ionic permeability, but associated with only a minimal decrease in the electrical capacity, which depends on ionic impermeability. Cole<sup>40</sup> has plausibly interpreted these findings as indicating that permeability to ions is confined to a relatively small part of the cell surface.

#### *Principle 5. Differential Changes in Permeability*

Space will permit the mention of only two examples of this principle. Several years ago, my assistant, Mr. Corson, happened to make up a glycerol solution in some commercial distilled water and, on using it with human erythrocytes, obtained a surprisingly low rate of penetration of the solute. We were later able to determine that this effect was due to traces of copper in the water and that it could be imitated by adding a copper salt to ordinary

distilled water in concentrations of  $10^{-5}$  M and lower. Further work has shown that under favorable conditions it can be obtained at concentrations down to the order of magnitude of  $10^{-8}$  M, and that it occurs with the erythrocytes of only a few of the many species of mammals so far investigated—of which those of man show it most clearly. It is also specific for glycerol. The only one of several dozen other substances studied that behaves at all similarly is monoacetin, which is a glycerol ester. The only other metal that closely resembles copper is mercury, but even it behaves differently in several important respects. Under favorable conditions, copper may decrease the permeability of human erythrocytes to glycerol to only 10 per cent of its original value, while leaving that to other hydrophilic non-electrolytes, to lipid-soluble substances, and even to anions essentially unchanged.

Not long after the discovery of this effect on the erythrocyte, Langmuir<sup>41</sup> reported a striking effect of copper in similarly low concentrations on monolayers of fatty acids. We were therefore tempted for a time to look to the lipids of the cell for an explanation of our effect. But we gave up the attempt because of two chief considerations. The first is that it is difficult to see how copper could change the physical properties of the cell lipids to the extent of almost abolishing glycerol permeability, without at the same time producing some parallel effect on their permeability to many other substances. The second is that all erythrocytes, and probably all other cells, seem to have lipid elements in their surfaces, but in only a few species is their permeability to glycerol strongly affected by copper. These, it turns out, are all species into whose erythrocytes glycerol enters faster than it should, *i.e.*, faster than would be predicted from its molecular volume and its low solubility in lipids. The copper effect seems to be very strong in the erythrocytes of the birds, mentioned above, into which glycerol enters as rapidly as ethylene glycol. Another reason for believing that the lipids are not primarily concerned in the copper effect will be discussed under principle 6 below.

By way of contrast with the specific effect of low concentrations of copper in decreasing the permeability of human erythrocytes to glycerol and leaving it almost unchanged to other non-electrolytes and anions, is that of tannic acid. This substance in low concentration strongly decreases the permeability to anions of the erythrocytes of several species so far studied, without at the same time, at body pH, much affecting their permeability to glycerol and other non-electrolytes. Tentatively, it seems plausible to suppose that the tannic acid acts upon the same protein regions whose permeability to ions is so profoundly altered by denaturation. The characteristic action of tannic acid on proteins is equally well shown in the tanning of leather and in its effects on protein monolayers.<sup>42</sup>

Space will not permit a discussion of the effect of narcotics on the permeability of the erythrocyte. It can only be said that this effect is highly complex. In low, non-injurious concentrations these substances seem typically to decrease the permeability of all erythrocytes to anions, to increase that of all erythrocytes to at least some lipid-soluble substances, but, in the

case of glycerol, to decrease it markedly in some species and not at all in others. Such a complexity of effects would appear to demand for its explanation an equal complexity of structure.

*Principle 6. Spatially Limited Permeability*

The effect of copper on the permeability of the human erythrocyte to glycerol has been mentioned. A quantitative study of it has yielded some suggestive results which must be incorporated in our hypothetical picture of the molecular structure of the surface of the erythrocyte. In a typical experiment, when approximately 250 million cells were exposed to 20 ml. of a glycerol solution containing a concentration of  $\text{CuCl}_2$  of  $6 \times 10^{-7}$  M, their permeability to glycerol was reduced to about 10 per cent of its original value. It is easy to calculate that, if in producing this effect all the copper atoms in the solution were taken up by the cells, there would be 30,000,000 of them for each cell. Other experiments have shown in several ways that the effect of the copper is produced at the cell surface rather than in its interior, and that it is almost instantaneous and completely reversible. From the known dimensions of the copper atom and of the human erythrocyte, it readily follows that, in the case just mentioned, the copper atoms could cover at most only a little more than 1 per cent of the total cell surface, despite the fact that they lower its permeability to glycerol by 90 per cent of its original value.

But this estimate is certainly too high. If erythrocytes are exposed to such a dilute copper solution and then quickly removed from it by centrifugalization, it can be shown that most of the copper remains in the solution rather than attached to the cells. In one such experiment, it was estimated that each cell could have taken up no more than 1,000,000 copper atoms, and these would suffice to cover no more than 1/2000 of its total surface. Figures such as these suggest that any effect of the copper on a number of fatty-acid molecules sufficient to form a bimolecular layer could hardly be expected to cause any very profound change in the physical behavior of such a layer.

In view of the highly specific nature of the copper effect for glycerol, in view of the fact that it appears only in erythrocytes which are "abnormally" permeable to glycerol, and in view of the further fact that spatially it appears to be confined to a very small part of the cell surface, it seems reasonable to suppose that copper produces its characteristic effect on the human erythrocyte by acting on some localized mechanism for glycerol transport not found in most erythrocytes. In favor of this view is the fact that, when maximally inhibited by copper, human erythrocytes still retain a rather low permeability for glycerol of about the same magnitude as that found in most other species of mammals, in which a molecular sieve mechanism presumably operates. It is tempting, therefore, to think that the special mechanism in question in the highly permeable cells is an enzyme. Support for this view is furnished by a recent short note by LeFevre,<sup>43</sup> who reports a similar specific inhibition of glycerol uptake by p-chloromercuribenzoate and other substances which, like copper, have an inhibiting effect on a number

of known enzymes containing -SH groups. Further details concerning this work will be awaited with interest, but in the meantime it should be noted that there is nothing improbable, or even new, about the idea that enzymes may be present in the cell surface. Tentatively, therefore, a place should be made for them in our hypothetical model.

The chief conclusion to be drawn from these studies can be summed up in a single word: "complexity." Simple models, such as that originally suggested, have their usefulness as a starting point for theoretical discussions. When they fail to account for all the known facts, however, they must be modified. If in the process they lose their simplicity, we need hardly feel surprised. Protoplasm in general is universally believed to have a very complex structure. Why should its strategically placed and enormously important surface be less complex in structure than its other portions?

A good deal has been made in the past of the fact that a complex mosaic type of structure of some sort, to which the known phenomena of cell permeability at present seem to point, is physically less stable than the model chosen for discussion. This difficulty does not seem to me to be a very serious one as far as the erythrocyte is concerned. In the first place, the erythrocyte surface, whether as a ghost or as a part of an intact cell, does not seem to have the properties of a simple liquid. There is some evidence that even its lipids may, at least in part, be in chemical combination with underlying proteins, which in turn may form a part of a continuous framework, or stroma. In the second place, whether or not stability is secured in this way, the fact of instability in itself presents no theoretical difficulties. Protoplasm is a notably unstable system, maintained only by a constant expenditure of energy. There is no logical reason why its surface should have fundamentally different properties.

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## THE ULTRASTRUCTURE OF THE ENVELOPE OF MAMMALIAN ERYTHROCYTES

By David F. Waugh

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.*

One can support the suggestion that most cells maintain a thin layer of surface material distinct from the underlying cytoplasm. This layer is to be distinguished from materials, such as cellular and cementing substance, which are considered as cellular secretion products. This thin layer, or plasma membrane, may be demonstrated by many techniques, such as the microdissection techniques of Chambers, optically by polarized light technique, and in other ways. With polarized light technique, the limiting material is distinguished, since it is doubly refracting and shows, in many cells, a distinct polarization cross. This method not only reveals molecular orientation in the plasma membrane but also in the nuclear membrane and in many other cellular components, such as chromosomes, the spindle and asters, mitochondria, *etc.*<sup>1</sup>

For those cells in which materials must pass through in a molecular state, it seems reasonable to suppose that the plasma membrane is the agency which allows differential passage or differential permeability. The eventual determination of the ultrastructure of the various plasma membranes should give a solution to this problem. In some cells, such as the proximal tubule cell of the kidney, a distinct limiting membrane is not demonstrable. In these cases the molecular structure of the plasma membrane may have little to do directly with "permeability," the entrance of materials into the cell being controlled in other ways, possibly by vacuole formation.

Although the techniques mentioned may demonstrate the presence of an outer layer having distinct molecular orientation, the presence of cytoplasm in close association has, in most cells, so far prevented a structural analysis. The mammalian erythrocyte presents an interesting exception. In these cells, as indicated by Jorpes,<sup>2</sup> and now generally agreed upon, the non-hemoglobin protein is present largely in the envelope. In addition, Erickson *et al.*<sup>3</sup> have found by chemical analysis that the total amount of lipid is also present in the envelope. This suggests that the internal cytoplasm of the red cell is small compared to the envelope material. This view is strengthened by the investigations of Fricke, Parker, and Ponder,<sup>4</sup> which indicate that the internal framework would not require more than about 0.1 per cent stromatin, a negligible amount compared to the envelope. Hemolysis serves to remove hemoglobin and salts. Where this can be done effectively, the envelope remains intact and may be studied by a variety of methods.

It seems obvious that the erythrocyte envelope may not be a typical "plasma membrane." In this respect, a complete analysis in which structural, permeability, and metabolic properties are correlated may not offer a general solution applicable to other cells—each may require separate analysis. Jacobs has shown, however, that the erythrocyte presents a challenging array of permeability problems. A solution to these should further our insight into similar situations presented by other cells.



**Polarization Optics.**<sup>1, 5</sup> A regular (crystalline) array of anisometric molecules, such as a fatty-acid crystal, has different properties in different directions. These include heat transfer, electrical polarization, cleavage, and effect on light. For present purposes, consider a fatty-acid crystal (FIGURE 1). A plane which includes cross sections of the chains (A) has no distinguishing directions, and light which enters the crystal perpendicular to this plane will emerge unchanged. This direction is termed the optic axis. Observation in a plane which includes the fatty-acid chains (B) reveals two distinguishing directions immediately. A beam of polarized light which enters the crystal perpendicular to this plane may or may not be altered as follows: If the plane of vibration of the polarized light is parallel or perpendicular to the fatty-acid chains, it will proceed unchanged. If the plane of polarization makes an angle with the fatty-acid chains, the incident beam will be broken into two components vibrating parallel and perpendicularly to the fatty-acid chains. The two beams thus produced will travel with different velocities in the crystal and may emerge out of phase. Thus the emergent beam is usually elliptically polarized. The difference in velocity of the

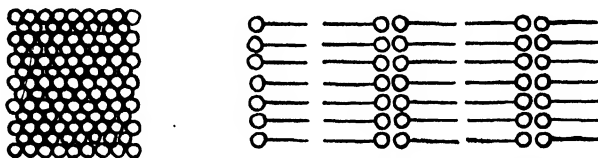


FIGURE 1. Schematic representation of a fatty-acid crystal whose chains are oriented perpendicularly to the planes of the carboxyl group. *Left (A):* a plane cutting at right angles to the hydrocarbon chains. *Right (B):* a plane cutting parallel to the chains.

two beams produced by the crystal means that the crystal has two refractive indices. One of the rays represented by these indices behaves according to Snell's law and is termed the "ordinary ray." The other does not and is termed the "extraordinary ray." The sign of the double refraction is obtained by subtracting the refractive index of the ordinary ray ( $N_o$ ) from that of the extraordinary ray ( $N_e$ ).

The fatty-acid crystal is a positive uniaxial crystal. The ray whose electric vector vibrates parallel to the fatty-acid chains (the direction of the optic axis in FIGURE 1) is the extraordinary ray, and the refractive index is greatest in this direction. The double refraction described is crystalline double refraction and is exhibited by most lipids and protein systems of oriented polypeptide chains. The optic axis lies parallel to the directions of the main molecular chains in both cases. Since biological materials are uniaxial, a discussion of more complicated types of crystals will be omitted. The presence of double refraction may be tested for by placing the object between two polarizing (Nicol) prisms. The prisms are oriented so that the polarized light produced by the first cannot pass through the second. The field is dark. If the object is oriented so that one looks down the optic axis, the field will remain dark on rotating the object. In directions other than the optic axis, the fatty-acid crystals will appear dark when the long direc-

tions of the chains are oriented parallel or perpendicular to the polarizing directions of the Nicol prisms. In other directions, the crystal will appear bright and will have maximum brightness at  $45^\circ$  orientation.

Double refraction is an intrinsic property which is independent of the size and shape of the system. The phase difference between the extraordinary and ordinary rays,  $\Gamma$ , is given by the product of double refraction and thickness,  $d$ . Thus:

$$\text{Double refraction} = n_e - n_o = \frac{\theta\lambda}{d} = \frac{\Gamma}{d},$$

where  $\theta$  is the phase difference and  $\lambda$  is the wavelength of light used. One ordinarily measures on retardation and divides this by thickness to get

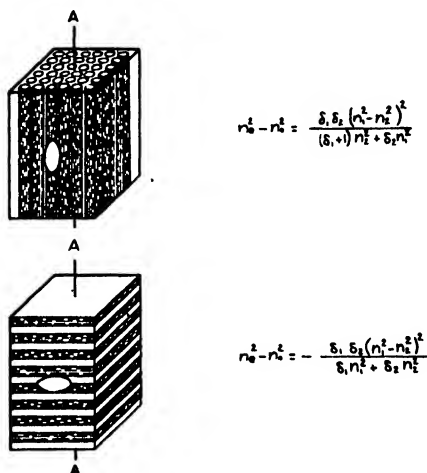


FIGURE 2. Rodlet and platelet form double refraction. The directions A indicate the optic axes. The corresponding Wiener equations are given on the right. Taken from Schmitt, J. Applied Physics 9: 109, 1938.

double refraction. A variety of compensators are available for determining signs of double refraction and retardation. For objects, such as the erythrocyte envelope, having little retardation, a thin mica plate having a total retardation of  $\lambda/20$  is placed in the optical path and used to compensate for the double refraction of the object. This is termed the Köhler compensator and, since its sign is known, the direction of rotation necessary for compensation gives the sign of double refraction of the material.

In addition to crystalline, double refraction systems of oriented rodlets or platelets, whose size is large with respect to the wavelength of light, will also show double refraction called "form" double refraction. O. Wiener, in 1912, calculated the behavior of such systems and derived formulae assuming that the particles have no crystalline double refraction of their own. For rodlets and platelets the situation is shown in FIGURE 2, which also shows the directions of the optic axes.

In these,  $\delta_1$  and  $\delta_2$  are the partial volumes of the particles and surrounding medium, respectively, and  $n_1$  and  $n_2$  are their refractive indices. Rodlet form double refraction is always positive, while platelet form double refraction is always negative. It is to be noted that form double refraction depends upon the difference between the refractive indices of particles and medium. If they are made equal by suitable media, the form double refraction disappears.

Most cellular materials have components of form double refraction and crystalline (or intrinsic) double refraction. According to the Ambrogn method, they may be separated, in instances, by immersing the material in a series of media of different refractive indices. FIGURE 3 indicates the

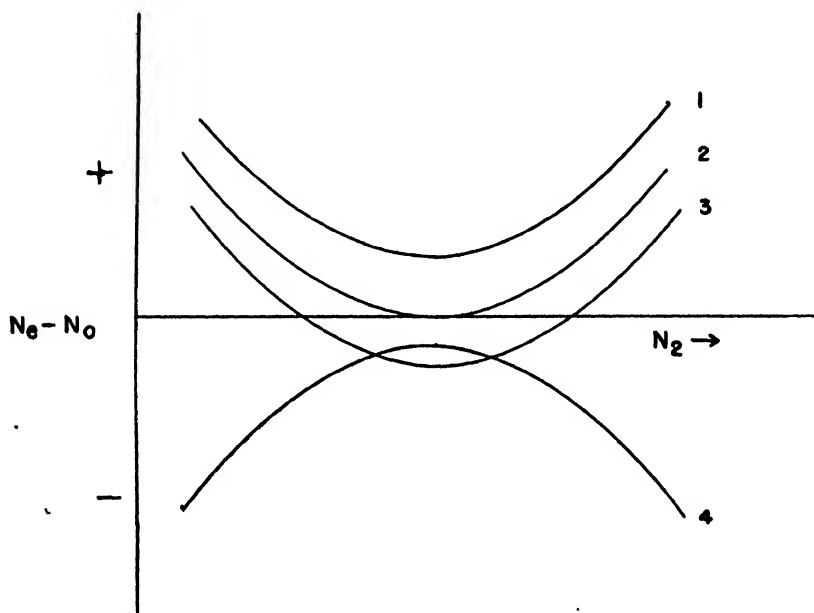


FIGURE 3. Dependence of double refraction on the refractive index of the immersion medium ( $N_2$ ). Curve 1, rodlet form double refraction and positive crystalline double refraction. Curve 2, rodlet form double refraction only. Curve 3, rodlet form double refraction and negative crystalline double refraction. Curve 4, platelet form double refraction and negative crystalline double refraction.

results where double refraction is plotted as ordinates against the refractive index of the immersion medium. The minimum in the hyperbolic curves occurs when form double refraction has been eliminated ( $n_1 = n_2$ ). This minimum, therefore, measures the refractive index of the particles if no complications occur. If the minimum occurs at 0 ordinate, the system has form double refraction entirely and the particles themselves are isotropic. If it is + or -, the double refraction at the minimum is the magnitude of crystalline double refraction due to the intrinsic structure of the particles. Form double refraction is generally associated with protein rodlets or platelets.

Normal red cells are too opaque to show polarization effects. After hemolysis by freezing and thawing according to Schmitt, Bear, and Ponder,<sup>6</sup>

and suspending in 1 per cent NaCl, the envelopes of rabbit cells show, if anything, a faint negative cross with the optic axis directed radially. After treatment with lipid solvents, the envelope has a definite form double refraction negative cross indicating protein leaflets or platelets tangentially oriented. Immersion in glycerol or urea solutions, which increase the refractive index of the immersion medium and decrease the protein form double refraction, immediately produces a positive cross with radially directed optic axis indicating oriented lipid, the long axes of the lipid molecules also being radially directed. The envelope appears, therefore, as a lipid-protein complex.

Since the thickness is below the resolving power of the microscope, the physical dimensions of the envelope do not permit one to calculate double refractions. Under normal circumstances, however, the form double refraction of the protein balances the intrinsic double refraction of the lipid component. The double refraction data establish with some certainty the orientations of the lipid and protein components in the envelope.

*The Electron Microscope.* A detailed discussion of the electron microscope is not necessary here. The instrument is familiar to most and the high degree of resolution, 50 Å, is present-day legend.<sup>7</sup> Wolpers<sup>8</sup> has prepared stroma by osmotic hemolysis followed by osmium fixation and estimates a membrane thickness of the order of 250 Å. If such cells are not fixed, the thickness is greater. He estimates the thickness in whole cells to be about 150 Å. Such figures are approximations only.

During the course of removal of lipid from osmotically hemolyzed cells, defects or holes appear in the envelope. If such extracted membranes are fixed with osmic acid, a fibrous structure is evident. The fibrils have widths of about 150 Å after this treatment. They may be considerably smaller *in vivo*, for the osmic acid may exert some condensing effect. Wolpers points out that the fibrous structure appears after the several steps in a rather drastic treatment: osmotic hemolysis, lipid extraction, osmium fixation, and high vacuum drying.

Wolpers views the envelope as being composed of a scaffolding or felt work of protein fibrils in which the lipid molecules are dispersed.

The fibrous nature of the protein component of the envelope was anticipated chemically by the work of Boehm in 1935.<sup>9</sup> Boehm dispersed stroma in lithium perchlorate and found that the resulting dispersion had a high viscosity and showed intense streaming double refraction.

The envelope material seems disposed to the formation of fibrous structures. Furchgott<sup>10</sup> treated suspended ghosts with lyotropic salts such as lithium perchlorate and potassium thiocyanate, much as Boehm had done, and found, using dark field technique, that the envelopes "unravelling" into long thread-like structures, networks of threads, or medusae. This is, apparently, a spontaneous reaction on the part of the ghosts, which can be lost by prolonged washing.

From evidence so far presented, it would appear that the protein leaflets responsible for negative form double refraction are composed of protein fibrils or filaments. Orientation or disorientation of fibrils within the plane of the protein leaflet remains to be demonstrated. Over large areas, there is

probably statistical disorientation.<sup>11</sup> Likewise the lateral extent, or average area, of the leaflets has not been determined. These results are in agreement with double refraction analysis. Double refraction does not specify the internal structure of the protein leaflet component.

**Reflectivity Method.** A direct measurement of the thickness and refractive index of the dried envelope before and after extraction with lipid solvents is possible with the leptoscope.<sup>12, 13</sup> Blodgett and Langmuir<sup>14</sup> and Blodgett<sup>15</sup> have made a careful analysis of the changes in intensity of light which is reflected from a glass surface covered by a thin transparent film. The variables involved are the refractive indices of film, supporting material, and medium (usually air) through which they are viewed, the angle of incidence and wavelength of the light, and the thickness of the film. By utilizing the

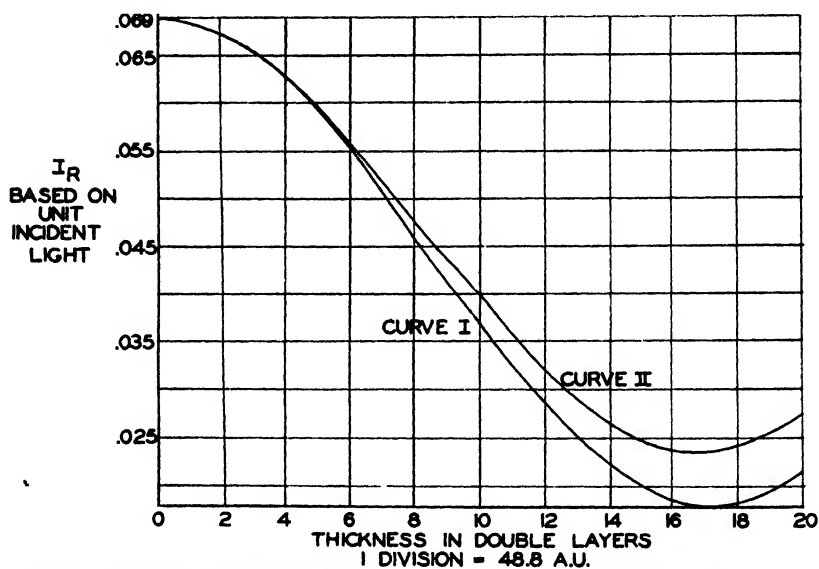


FIGURE 4. Reflectivity of a glass of  $N_g = 1.70$  at  $\lambda = 5,000$ . The curves are based on unit normal incident intensity. Curve I refers to a film of barium stearate having a refractive index of 1.495, while Curve II refers to a film (possibly protein) having a refractive index of 1.525.

film-building technique of Langmuir and Blodgett, in which a plate is dipped through a monolayer of mixed stearate and stearic acid under pressure, one can deposit a film having *steps* of 48.8 A.U. thickness increment. These films have a refractive index of 1.495 and, when viewed at vertical incidence, will conform to Curve I of FIGURE 4. The wavelength of the light is assumed to be 5000 A.U. and the refractive index of the glass 1.70. The ordinates are based on unit incident intensity and the abscissa represents film thickness in double layers. Curve II represents a film having a refractive index 1.525 under the same conditions. One observes that the intensity-thickness curve is a cosine squared curve, that the change in intensity for a given small thickness increment is largest over the central portion of the curve, and that the first minima occur at about 17 double layers or 830 A.U.

The difference of intensity between maxima and minima depends upon the difference in refractive index between film and glass. This should be as large as possible.

By using a set of glass plates covering a range of refractive indices in small increments, one can measure the refractive index of a film by noting that glass on which all thicknesses of film reflect the same intensity of light as the clean glass. This serves as a convenient method for measuring the refractive indices of materials in micro quantities ( $10^{-10}$  gm.) or in quantities which, though still small, are large enough to yield a film visible to the eye.

These observations may be applied to biologic membranes by depositing such membranes on a glass of high refractive index and observing them with a suitable technique. The technique used previously, and one which is

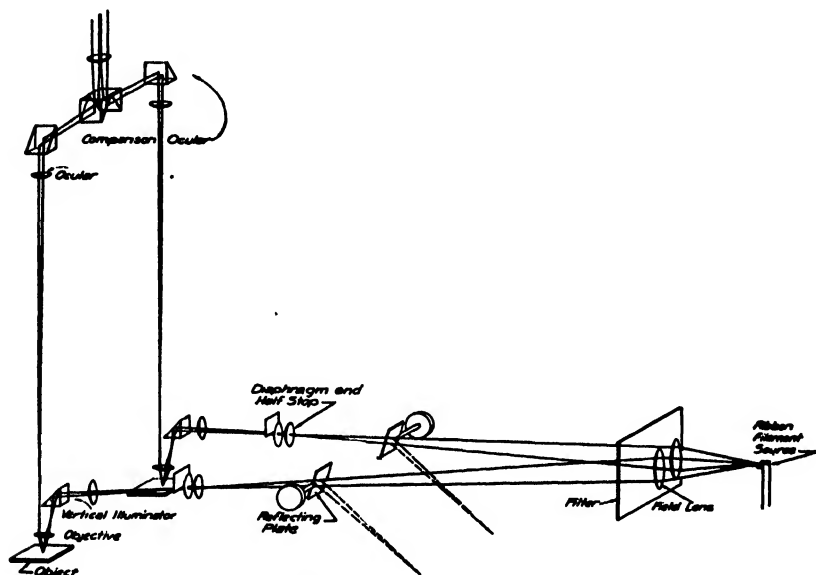


FIGURE 5. Optical system of the leptoscope.

under modification at present, is shown in FIGURE 5. For obvious reasons, the technique utilizes microscopes. Since *reflected* light is to be observed, one resorts to the vertical illuminator, a device in which the light which enters horizontally is reflected downward to the objective. The objective focuses the light on the object which reflects a portion back to the objective. At this time, the objective serves its normal function. It picks up the reflected light and focuses it toward the objective. The system used is a double one having two microscopes and two vertical illuminators with a single source of light. The envelope preparation is made on one slide and is placed on the microscope. The other microscope accommodates a built-up film of barium stearate having a number of steps from 0 on up, each differing by a single double layer of stearate. The comparison ocular views one half of the field from each microscope, thus allowing a direct comparison to

be made between the dried envelopes and a film of known thickness. For details see References 12 and 13.

The envelopes are prepared as follows. While in a horizontal position, a clean slide is covered with a few ml. of a highly dilute suspension of washed red cells. During the next two minutes, some of the cells will settle and adhere to the slide. The excess cells are washed off with isotonic saline; the slide is drained and then immersed in a  $10^{-4}$  M buffer made with double distilled water. The dilute buffer hemolyzes the cells. While the slide is immersed, the surface of the hemolyzing buffer is covered with a monolayer of egg albumin or other protein. After 1 minute, the slide is then dried in a vertical position. Drying takes about 2 minutes. This time is considered as being part of the hemolysis time. The protein monolayer lowers the

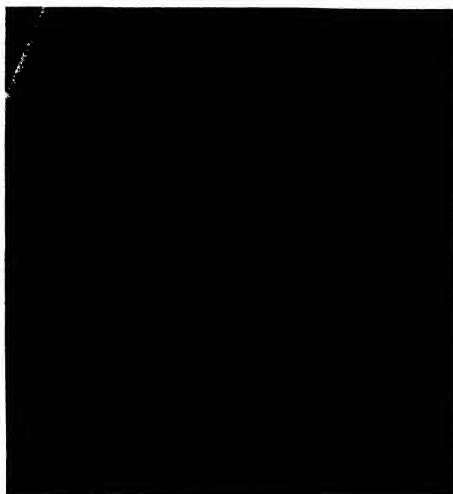


FIGURE 6. View through comparison ocular of leptoscope. Double envelope discs are shown on left. On right is a step film showing, above, 6 double layers and, below, 7 double layers.

surface tension of water sufficiently to prevent disruption of the envelopes when they reach the interface. There is sufficient residual tension, however, to draw them into two layered discs and they dry as such, as shown in FIGURE 6. This is a photograph of the field through the ocular of the leptoscope showing a film of 7 and 6 double layers on one side and envelope discs of the rabbit on the other. To measure thickness one moves the built-up film until a matching step or interstep is obtained. The known thickness of this step and the refractive index of the envelope are used to calculate actual envelope thickness. The refractive indices of most unextracted envelopes are sufficiently close to the film refractive index to require no correction.

The slide with the dried envelopes may be dipped in lipid solvents and material thus extracted. A remeasurement of refractive index and thickness now gives a measurement of the material taken out of the envelope.

The dried envelopes have varying diameters. The measured thicknesses thus are usually not the thicknesses of the envelope *in vivo*. Comparable thickness values for different species may be obtained by calculating the volume of material in the envelope disc and dividing this value by the area of the erythrocyte *in vivo*. Values given by Ponder in his monograph of 1934<sup>16</sup> have been used. The values are rabbit  $106\mu^2$ , rat  $106\mu^2$ , and steer  $63\mu^2$ .

The results for rabbit erythrocytes are typical (FIGURE 7). In this, the thickness of the envelope in A.U. is plotted as ordinate with the pH of the hemolyzing buffer as abscissa. The maximum normal thickness occurs at pH 6.0 and is about  $215 \text{ \AA} \pm 15 \text{ \AA}$ . On either side of this pH, the thickness decreases rapidly indicating loss of material. These same cells, leached in a mixture of chloroform and alcohol (2:1) brought to boiling for 15 min., gave

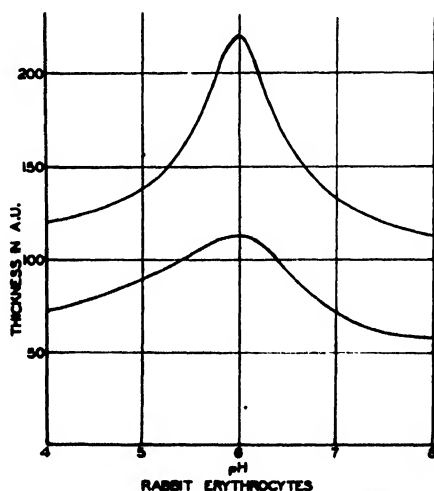


FIGURE 7. Thickness in Angstrom units of erythrocyte membrane of rabbit cells versus the pH of the hemolyzing buffer.

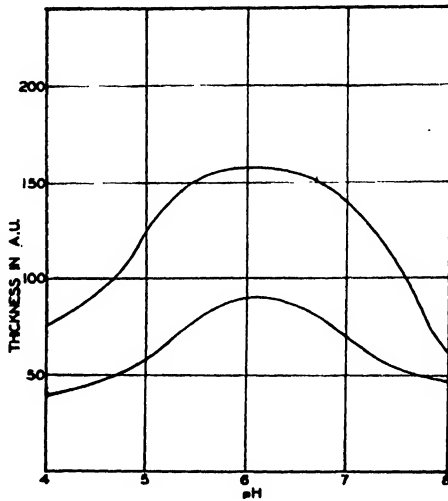
thicknesses as shown in the lower curve. The composition of the envelope seems relatively constant, indicating that the disruption by hemolysis removes components of constant composition and not protein or lipid soluble materials selectively. The thickness of leached cells is about 110 A.U., giving a volume ratio of lipid to protein of 1 to 1 or a weight ratio of 0.7 (assuming lipid density of 0.9 and protein density of 1.3).

Much the same situation is presented by rat and steer cells. The curves obtained with rat cells (FIGURE 8) shows that these cells, over a hemolyzing range of pH 5.5–6.7, are relatively stable compared to rabbit cells. Here, the total maximum thickness is 160 A.U., the leached thickness being about 90 A.U., thus, a volume ratio lipid to protein of about 0.8. Again, disruption of the envelope by the hemolyzing buffer indicates the envelope to be losing material of a fairly constant composition.

Steer cells (FIGURE 9) are considerably different from the others. In

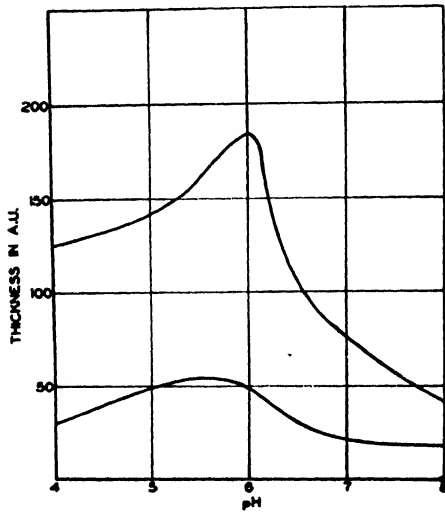


these, the maximum thickness, at pH 6.0, is 180 A.U., the leached thickness being about 60.0 A.U., a volume ratio of lipid to protein of 2:1. In addition,



RAT ERYTHROCYTES

FIGURE 8. Thickness in Ångstrom units of erythrocyte membrane of rat cells versus pH of the hemolysing buffer.



STEER ERYTHROCYTES

FIGURE 9. Thickness in Ångstrom units of erythrocyte membrane of steer cells versus pH of the hemolysing buffer.

steer cells are less resistant to alkaline buffers than to those on the acid side of the pH of maximum stability. This may be associated with the larger amount of lipid extractable material.

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A summary of the data for rabbit, rat, and steer cells is shown in TABLE 1. It should be remembered that the recorded pH of maximal stability is that of the hemolyzing buffer. The pH in the neighborhood of the envelope may differ considerably from this value.

In the case of rabbit cells, on which more experimental work has been done than on the others, certain types of behavior are to be described.

As shown in the previous figures, the envelope is unstable in the absence of electrolyte. In the presence of isotonic saline, however, red cells may be buffered at pH of 5.0-8.0 without loss of material from the envelope. The

TABLE 1  
THICKNESS AND COMPOSITION OF THE LIMITING ENVELOPE OF MAMMALIAN ERYTHROCYTES

Animal	Maximal Thickness						Lipid: Protein Ratio	
	pH	Total Envelope	Lipid		Protein		By Volume	By Weight
		AU	AU	% of Total	AU	% of Total		
Rat	5.7	160	80	50	80	50	1.0	0.60
Rabbit	6.1	215	135	63	80	37	1.7	1.01
Steer	6.1	180	125	69	55	31	2.3	1.36

TABLE 2  
EFFECT OF ABSENCE OF ELECTROLYTES ON THE LIMITING ENVELOPES OF RABBIT ERYTHROCYTES

pH	Time in hemolyzing solution	Thickness			Refractive Index	
		Total envelope	Extracted envelope	Difference	Total envelope	Extracted envelope
	min.	A	A	A		
6.1	1	232	131	101	1.504	1.525
6.1	2	196	113	83		
6.1	5	203	110	93		
6.1	10	146	86	60		
6.1	20	116	86	30	1.517	1.525
6.1	40	ca. 120	ca. 90	30		
		130	CO <sub>2</sub> Ghosts 100	30	1.520	1.525

addition of  $10^{-6}$  M copper ion to the cells before and during hemolysis likewise reduces pH sensitivity. In addition, the ability to extract material with lipid solvents disappears. The copper evidently integrates the structure of the envelope by cross linking. Structural changes of this sort are probably the basis of the permeability changes found by Jacobs and Corson<sup>17</sup> and Davson and Danielli<sup>18</sup> for envelopes treated with heavy metal ions.

There is a general correlation between refractive index of envelopes, hemolysis time, and materials lost from the envelope at the hemolyzing pH of maximum thickness. TABLE 2 shows a summary of this information.

The first line refers to the standard technique. The envelopes have a thickness of 232 Å and a refractive index of 1.504. After leaching, the thickness decreases by 110 Å and the index of refraction rises to 1.525. Simple calculation indicates that the material leached out has a refractive index of 1.490, quite close to the refractive index for lipid materials. If cells are allowed to stand in the hemolyzing buffer for longer periods of time, the total thickness decreases and, at the same time, there is a preferential loss of low refractive index material from the envelope. The envelope is relatively stable with 30 Å of extractable material still present. During this time the extracted thicknesses decrease by 45 Å (Column 4), indicating the loss of protein. The envelope, air dried, is essentially a dehydrated structure, for, after several hours at 100°C, the thickness loss is less than 5 per cent. Thus, the thickness changes described are not due to changes in hydration.

The water content of the envelope *in vivo* is of more importance in determining permeability properties. The leptoscope adds information to this problem. Observation during drying and particularly just after recession of the water meniscus indicates that the envelope in the hemolyzing buffer, and probably therefore *in vivo*, is a structure containing less than 25 per cent water dispersed throughout its structure. This is in rough agreement with polarization optics. Thus, the *in vivo* envelope may be considered as a desolvated structure in which the component molecules are in close association.

In many instances, special structure may be seen in the envelope discs (see 12, Figure 2 B). Here, the central part of the discs reflects less light than its surroundings, indicating a thickening in this region. The difference in thickness is accentuated on leaching, showing that the thickening is due mainly to protein. One would expect this region to correspond to the regions of the biconcavities of the erythrocyte, for the cell adheres to the slide with a maximum surface in contact, thus with the concavities down. These protein thickenings in the regions of the biconcavities suggest that the shape of the cell is determined structurally in the envelope. The envelope, therefore, may have different molecular structures over its surface, not in the sense of a mosaic of submicroscopic regions but in the sense of regions, each of which constitutes a fair percentage of the entire cell surface.

Ponder in 1942<sup>19</sup> described the events leading to osmotic hemolysis and post-hemolysis phenomena as follows. The cells increase in volume, losing their biconcavities (stepwise?), and become spherical after an increase in volume of about 60 per cent. At this point, the hemoglobin escapes and the cells fade, but retain some hemoglobin. If the cells are now returned to isotonic saline, they shrink at first and then return to the biconcave disc structure. It seems improbable that internal strands of material, bridging the gap between the concave portions, could undergo such large changes in length in a reversible fashion.

**Chemical Analysis.** Chemical analyses of the lipid content of carefully washed hemolyzed cells, and in instances whole red cells, have been performed by a number of investigators (for a review see 20). With few exceptions, chemical analyses are in good agreement as to the total amount of

material present which may be extracted by organic solvents. The groups differ somewhat as to the distribution of this total lipid among the various lipid fractions, although the bulk of the material is accounted for by cephalin, cholesterol, and cholesterol esters. Dziemian<sup>21</sup> has made an extensive investigation of normal rabbit cells, Erickson *et al.*<sup>8</sup> have examined bovine and sheep cells, and Ponder<sup>22</sup> human erythrocytes. All agree that the content of lipid is sufficient to cover the surface of the cell with a layer about 40 Å thick. Erickson *et al.* have found only small amounts of lipid in the filtrate from hemolyzed cells, which is cited as evidence that no lipid is lost during hemolysis.

This value of 40 Å for rabbit cells (Dziemian) is to be compared with the 100 Å of lipid or extractable material found by the leptoscope. The difference is large enough to necessitate a careful re-examination of the materials which may be present in the envelope and of the various methods used to determine thickness. The leptoscope is currently being modified to give more accurate and completely objective analyses on single cells.

It is of consequence that the main phospholipid of the envelope is cephalin, a compound having a low iso-electric point and therefore capable of binding basic proteins with strong linkages, as Chargaff and co-workers<sup>23</sup> have shown.

The protein moiety has received attention. Jorpes<sup>24</sup> originally showed that the envelope protein from several sources contained 5.8 per cent arginine, 2.63 per cent histidine, 3.01 per cent tyrosine, and 1.46 per cent tryptophane on a dry weight basis. He found that the protein has a flocculation point at pH 5.5 and is therefore acidic. He concluded that the stroma-protein belonged to no well-defined group and therefore was a new type of protein. More recently Beach *et al.*<sup>25</sup> examined beef, sheep, hog, horse, and human erythrocytes. They found no significant differences between them, in agreement with Jorpes. Their analyses for beef cells gave: 1.9 per cent histidine, 5.1 per cent arginine, 3.5 per cent lysine, 2.9 per cent tyrosine, 1.2 per cent tryptophane, 0.78 per cent cystine, 1.5 per cent methionine, and 0.708 per cent total sulfur. To these values we may add those of Ballentine<sup>26</sup> for beef as follows: 3.66 per cent glycine, 10.7 per cent leucine, 3.42 per cent tyrosine, 1.45 per cent tryptophane. Ballentine also examined sheep and human cells and found no appreciable differences. From the content of leucine and glycine, he agrees with Jorpes that stromatin represents a new type of protein at present peculiar to the erythrocyte envelope. The possibility that more soluble proteins are being washed out during preparation is recognized.

Chemical analyses agree, therefore, that the stromatin recovered from a number of mammalian species after prolonged washing has a relatively constant composition and probably represents a characteristic envelope protein. A correlation between permeability properties and the lipid or protein content of the envelope has not been made.

Rough calculations, based on Jorpes<sup>24</sup> calculations that about 4 per cent of the total erythrocyte protein is stroma protein and Ballentine's<sup>26</sup> recovery of 14-16 gms. stromatin from 2 liters of packed washed cells, indicate that

the thickness of the protein component of the washed envelope is approximately 70 Å.

*Status of Molecules within Membranes.* We have seen that the results of polarized light and electron microscope investigations suggest the protein component to be in the form of platelets, each platelet being composed, in turn, of a feltwork of filaments or fibrils. The average thickness and lateral domain of these platelets remains to be determined. The lipid molecules also remain undetermined in the sense that we know only that they must be radially oriented and that they probably exist in groups to account for the intrinsic double refraction. The unique properties of the envelope may be encompassed in a structure having a maximum dehydrated thickness of 215 Å and a hydrated thickness only about 25 per cent more than this value. Further insight into the problem may be gained, however, if we admit evidence from other sources as bearing on the problem. A careful investigation of the structure of the nerve myelin sheath, a lipid protein complex, has been made by F. O. Schmitt, W. J. Schmidt, R. S. Bear, K. Palmer, and others (see Reference 1), using polarized light and X-ray diffraction techniques. The situation may be summarized as follows: Polarization optics show the sheath to be constructed of concentric layers of protein with layers of lipid in between. The lipid molecules have their long axes radially oriented. X-ray diffraction evidence shows, in the radial direction, a fundamental repeat period of 170–180 Å, with other spacings of 4.7 and 9.4 Å, considered as due to the lipid side chains and whole molecules. These latter spacings suggest that the lipid molecules are closely packed in planes and exist as a mixed phase. The long spacing of 170–180 Å is sufficient to accommodate approximately two double layers of lipid, leaving 25 Å for protein. The position of the protein in nerve has not been determined. It may exist in between each double layer of lipid or, more probably, in between each second double layer of lipid.

The normal association of polar groups with polar groups and hydrophobic groups with hydrophobic groups causes lipid to associate, typically, in double layers. This tendency is evidenced strikingly in built-up films, as shown previously, and is evidenced also in emulsions of several varieties of lipid molecules. Hess, Philippoff and Kiessig, Stauff, and Kiessig and Philippoff,<sup>27</sup> working with sodium oleate solutions, and Schmitt and Palmer,<sup>28</sup> with nerve and other lipid, found this to be a general rule. Indeed, the double layer structure is maintained even though the planes of polar groups are expanded by intercalation of water<sup>27, 28</sup> or the planes of the hydrophobic residues are moved apart by the intercalation of organic solvents.<sup>27c</sup> In the former case a condensed structure with polar groups in close apposition may be restored by the addition of 0.6 M KCl or as little as 0.04 M CaCl<sub>2</sub>.<sup>28</sup> The striking effect of calcium is probably due to its ability to bind polar groups of two contiguous layers. As Schmitt and Palmer point out, the presence of such salts in biologic systems would cause the lipid layers to be held in close association with a minimal amount of water between them.

On the basis of such evidence, it seems reasonable to suppose that the lipid of the erythrocyte envelope is present, at least in local regions, in condensed layers, possibly double layers. The structure of the envelope may

not, however, conform to that found for the myelin sheath of nerve. The questions associated with mosaic structure, uniform complexes, *etc.*, are as yet unanswered.

**Monomolecular Films.** Evidence drawn from monomolecular film work indicates certain molecular mechanisms which may play an important part in determining permeability properties of cells and foreshadows the possible degree of complexity with which ultrastructure determination may have to deal. For some time, it has been known that a compressed film of stearic acid on a water surface at low pH produced little measurable retardation of evaporation of water, while a film of cetyl alcohol would reduce evaporation by one half. Langmuir and Schaefer<sup>29</sup> have recently analyzed the process in considerable detail. They suspend a drying agent over the film and measure the increase in weight of the drier with time, thus enabling a quantitative calculation of the resistance offered by the film. Langmuir and Schaefer show that the presence of the film probably introduces an energy barrier to evaporation. The extent of this energy barrier is largely determined by the internal stresses which occur within the monolayer. As they point out, the water surface holds the polar groups rigidly within a plane, the hydrocarbon chains lying all on one side of the plane. The hydrocarbon chains have minimal energy values for specific angles of tilt with respect to the basal plane. From the known properties of hydrocarbons these angles are  $90^\circ$ ,  $63^\circ 10'$ , and  $44^\circ 40'$ . At the same time, the areas occupied at the chain ends are correspondingly 18.5, 20.51, and  $26.0 \text{ \AA}^2$ . Between the minima in potential energy corresponding to these areas must be maxima.

The polar groups tend to spread out over the surface, while a contracting force is represented by the attractive forces between hydrocarbon tails. The measured surface pressure is the resultant of these forces.

The area per molecule must be the same for head and tail. If the heads are forced apart and must occupy areas somewhat larger than those corresponding to minima, *e.g.*,  $20.5 \text{ \AA}^2$ , the tails must do likewise. This increase in area must give rise to large contracting forces which oppose the increase in area. Langmuir and Schaefer show, however, that this effect alone is not sufficient to account for the energy barrier. The remainder is to be accounted for by the crystal-like properties of condensed monolayers. The presence of a water molecule between the heads causes a local breakdown in crystal structure which affects a relatively large number of neighboring molecules. Many molecules co-operate in producing the barrier and in this way the tremendous forces opposing passage may be accounted for.

The layers of lipid molecules in cellular elements have a structure corresponding closely to that of condensed films on water. One would expect the same general principles, modified as to detail, to apply in both cases. Thus one might conclude that the energy barrier associated with permeability<sup>30</sup> is due to the specific packing of the component molecules (protein as well as lipid). It is likely that this packing is determined with great precision to account for the specificities involved.

It is interesting to note that the great resistance to evaporation offered by cetyl alcohol monolayers is profoundly altered by the presence of minute

amounts of materials having a much lower resistance. The obvious inference is that small amounts of surface active materials change cellular permeability by a similar mechanism.

The greater part of the discussion presented has been drawn from material now several years old. The author is aware that no mention has been made of the dynamic aspect of the cell surface, the changes in structure which may occur momentarily and which are undoubtedly integrated with the simultaneous activity of the cell. We can hope that new instruments and techniques will be developed in the near future for handling the next phase of ultrastructure determination: the many problems associated with the interaction of molecules, particularly those interactions which depend upon specific intermolecular forces. This end may best be accomplished by integrating information coming from biochemistry and the application of physical tools to biological problems.

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### *Discussion of the Paper*

DR. MAURICE M. BLACK (*Brooklyn, New York*): Polarization optics, leptoscopic examination, and electron microscopy constitute valuable tools in the elucidation of the structural organization of biological material. However, it should be kept in mind that manipulative procedures incident to these modalities may often introduce extensive artifacts. Thus, the discrepancies in the determination of the structure of the red-cell membrane by different investigators using somewhat different methods may be explained on alterations incident to examination. The lability of lipo-protein systems is well demonstrated in the myelin sheath of nerve fibers. I have done some work on isolated peripheral nerves<sup>1, 2</sup> and find that immersion of freshly prepared nerve segments in water or non-electrolytes results in a swelling of the myelin and movement of the latter out of the cut ends of the fiber. This is associated with a loss of the birefringence of the myelin sheath. A definite relation exists between the pH of the solution and the amount of the reaction observed, no visible reaction occurring at pH 4.8 but increasing to maxima at the extremes of the pH scale. Instability of the lipo-protein complex is further demonstrated by the fact that undue handling causes myelin-forms to be produced in the sheath. Moreover, the lipo-protein orientation is upset as evidenced by the fact that the handled parts do not possess the double refraction found in the rest of the sheath. Thus, it is clear that lipo-protein orientation must be in an extremely delicate balance. Similar alteration in structural orientation was found in the axis cylinder, where even such slight injury as produced by immersion for several hours in isosmotic saline solution will result in loss of previously demonstrable neurofibril structure. Every one agrees that such lability must be considered a limiting factor in measurements designed to evaluate structural relations in biological material.



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DR. RUDOLF HÖBER (*Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pa.*): It is suggestive to test with erythrocytes the pore theory and the solvent theory of permeability by measuring the membrane potentials. The erythrocytes of some Amphibia (e.g. *Proteus*) have a diameter up to 50 to 70  $\mu$ . This is comparable to the thickness of single muscle fibers of frog (70  $\mu$ ) which have been shown by Graham and Gerard to allow puncturing of the plasma membrane with capillary microelectrodes (5  $\mu$ ). Possibly it will be very useful to study in this manner the effect of neutral non-toxic, non-polar-polar organic sodium salts having anions of various sizes.

DR. ERIC PONDER (*Nassau Hospital, Mineola, New York*): It is very satisfactory to workers in the field to see, from the contents of Dr. Jacobs' and of Dr. Waugh's papers, that there is so much agreement regarding the essential architecture and properties of the red-cell surface ultrastructure. It seem to me, however, that the old and still not wholly settled controversy, as to whether the mammalian red cell is a balloon-like body with a fluid interior or a gelatin-lozenge-like body with an internal structure, is reappearing in a new form. For we now have to ask ourselves: How do we know that some of the properties which we ascribe to the surface, such as some of the permeability properties, are not really surface properties as modified by the properties of the cell interior, or even properties largely dependent on what is on the inside of the surface ultrastructure?

In the case of many changes which happen rapidly, such as the transfer of water, of anions, and of many organic substances, and also in the case of the volume changes which these rapid transfers produce, the effects of the interior can probably be ignored, because it is likely that they enter into the equations which describe these rapid happenings only in such a way as to change the values of constants. There are situations, however, in which there is evidence that the interior of the cell, with its closely packed and probably oriented Hb molecules, plays an important part in the phenomena observed. The departure from ideal osmotic behavior shown by crenated red cells seems to be associated with the paracrystalline state of their Hb, and this may be so extreme, as in the rat red cell which has been kept in isotonic citrate in the cold, that the cells do not hemolyze or even swell when placed in distilled water. Another phenomenon, which is probably dependent on forces associated with the cell interior, is the slow escape of cations from red cells which have been exposed to hypolytic concentrations of lysins, and particularly to hypolytic concentrations of alcohols such as resorcinol, in which human red cells may exchange more than 50 p.c. of their K for Na and yet undergo less than a 10 p.c. increase in volume. It is true that these phenomena are observed only under special conditions, but they, as well as the properties of normal red cells, have to be accounted for in any complete description of red-cell structure. Otherwise, the description would be like a description of the structure of muscle which could account for the resting state and for contraction, but not for a phenomenon such as contracture.

It seems to me that it is possible to accept most of the information regarding the properties of the red-cell surface which has been accumulated, but not to be content with the description until a fuller account has been taken of the group of phenomena which seem to depend on the properties of the material which the surface ultrastructure encloses. Until matters are clarified, one should not allow one's self to think that when one measures  $dQ/dt$ , the rate of passage of a substance across the surface of the cell, one is necessarily measuring permeability properties confined to a membrane or even to a surface ultrastructure. It is possible that in some cases one is measuring the rate at which a material distributes itself between the suspension medium as one phase and the material of the cell surface plus the material of the cell interior as another phase, without the measurement's having any simple relation to membrane permeability as ordinarily understood.

Dr. Chambers' reference to a surface structure which may have no inner boundary, but which passes into the structure of a cytoplasm within it, expresses an idea which appeals to me strongly, as also does his description of the general nature of the cytolytic process. A process of the same kind may be involved in lysis of the red cell. Wilbrandt's colloid-osmotic hemolysis, which is similar to the "dual mechanism" hypothesis which Davson and I advanced in 1938, is certainly not satisfactory as a complete account of what happens. Both the colloid-osmotic hypothesis and the dual mechanism suppose that lysins render the red cell permeable to cations, and that this is necessarily followed by a secondary Donnan swelling of the cell. There are many instances, however, in which mammalian red cells slowly become permeable to both K and Na, but in which large ionic exchanges occur without more than a small volume increase and without loss of stability, *i.e.*, without the secondary Donnan swelling. One gets the impression that the full effects of the osmotic pressure of the intracellular Hb are not observed until some new event takes place. This event probably follows the K-Na exchange and is an immediate antecedent of lysis. It may be a cytolytic process, initiated by the collapse of the surface ultrastructure, and spreading through an interior composed of oriented Hb and stromatin molecules.

# STUDIES ON CELL MORPHOLOGY AND FUNCTIONS: METHODS AND RESULTS

By Albert Claude\*

*The Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y.*

To a large extent, our present knowledge of cell morphology has derived from observations of cells and tissues under the microscope, but inquiries by the microscopical method alone have repeatedly failed to give conclusive information on the chemical constitution of the various cell structures and the topography of biochemical functions.

The chief difficulties inherent in histochemical methods are: (1) the chemical tests which otherwise may be specific must be carried out on cell structures altered by fixation and dehydration to an extent which cannot be ascertained; (2) the chemical reactions studied must take place on structures, and in surroundings, of unknown and uncontrollable complexity; and (3) at the microscopical level, one must deal with exceedingly small amounts of substances, which often places the tests beyond the sensitivity of the reaction to be employed.

Since Miescher, attempts have been made to apply analytical methods to the study of cell structures and to isolate known cell components mechanically or through mild chemical manipulations. Separation of nuclei led to the discovery of nucleic acids and the recognition of certain basic proteins.<sup>1</sup> In 1913, Warburg segregated cytoplasmic granules by centrifugation and was able to show that these were responsible for most of the oxygen uptake of cell-free extracts of guinea-pig liver.<sup>2</sup> In 1934, Bensley and Hoerr succeeded in separating, also by centrifugation, large cellular elements which they identified as mitochondria.<sup>3</sup> In 1938, submicroscopic components of the ground substance were isolated by means of centrifugation at high speed.<sup>4</sup> These results indicated that, given the proper equipment and a systematic application of the method of differential centrifugation, it would be possible to separate the various cell components by mechanical means.<sup>5-8</sup>

In this paper, I shall attempt to review and discuss briefly the usefulness and the difficulties of the method.

*The Method of Differential Centrifugation.* The method of fractionation by differential centrifugation, as applied to cell studies, precisely overcomes the most serious limitations of the microscopical technique. By means of centrifugation, the whole range of particle and molecular sizes can be investigated, since centrifuges can be made to provide centrifugal fields of practically any given strength. In practice, however, the use of very high centrifugal force is not necessary. Under ordinary conditions, *i.e.*, when the viscosity and density of the medium is approximately that of water or saline, the cellular components with a diameter equal to, or greater than, 50  $\mu$  can be separated completely by centrifugation of one hour at 20,000 *xg*. In this manner, particulate components of cytoplasm (microsomes) too small to be detected by light microscopy could be demonstrated and isolated in

\*Present address: Institut Jules Bordet, 1 Rue Heger Bordet, Brussels, Belgium.

bulk.<sup>4, 7</sup> The second outstanding advantage of the method of differential centrifugation is that cell components can be obtained in practically unlimited amounts, thus making it possible to investigate their constitution and activities by the use of current chemical and biochemical methods.

Providing that the separation of the cell elements has been effective, a fact to be ascertained by suitable microscopical methods, the observations cannot take their full significance, and indeed can be misleading, unless the chemical and biochemical tests are carried out on a strict quantitative basis and the values pertaining to each individual fraction estimated in terms of the total capacity of the unfractionated cell and that of the other cell portions. For example, the microsome fraction was found to exhibit certain enzymatic activities (catalase; succinoxidase), but these had to be considered negligible in view of the considerably greater activity possessed by the whole suspension and by the large granule fraction.<sup>9</sup> Likewise, the distribution of ribose nucleic acid should be re-examined on a more quantitative basis, since ribose nucleotides are among the most common constituents of cells and, without special care, may be found in every fraction isolated.

*Three Main Cytoplasmic Fractions.* Under given conditions, it is possible to break a certain proportion of tissue cells while the free nuclei remain intact.<sup>8</sup> The debris, unbroken cells, and free nuclei can then be removed by low-speed centrifugation, leaving a suspension which contains essentially the components of the cytoplasm. By centrifugation at appropriate speeds, this "cytoplasmic extract" can be fractionated, stepwise, into three main portions: (a) a *large granule* fraction composed of elements of relatively large size, *i.e.*, ranging roughly from 0.5 to 2.0  $\mu$  in diameter; (b) a *microsome* fraction consisting in "sub-microscopic" elements approximately 50 to 200  $m\mu$  in diameter; and (c) the *supernate*, left after the two preceding fractions have been removed. This fraction contains the constituents of the cytoplasmic extract not sedimentable at the speed employed and, therefore, inferior in diameter to 50  $m\mu$ .<sup>8</sup>

It must be pointed out that the division of the cytoplasmic extract in this manner is not arbitrary and is not based on size differences alone but is determined by the fact that each of the three fractions isolated possesses physical properties, a chemical constitution, and biochemical activities of its own.

The so-called large granule fraction consists of small elements limited by what appears to be a semi-permeable membrane, which is found to respond osmotically to variations in the salt concentration of the medium. Thus, the large granules can be observed to swell or shrink when the salt concentration is lowered or increased.<sup>7</sup> The existence of a membrane has been demonstrated by electron microscopy.<sup>10, 11</sup> When concentrated in the centrifuge, the large granules give a mass which is opaque, brown, or distinctly yellow.<sup>3, 12</sup> Chemically, the major constituents of the large granules are proteins, phospholipids, ribose nucleic acid, ribose nucleotides, and flavins. The most distinctive feature, however, is that the large granules contain a number of enzymes and enzyme systems which are not found in the other cytoplasmic fractions. For example, the enzyme d-amino acid oxidase and the corresponding co-enzyme were found to be associated entirely with the

large granules, the other two fractions exhibiting no activity. Likewise, conclusive evidence has been obtained which indicates that important parts of the respiratory system, namely, cytochrome oxidase, succinoxidase, and cytochrome c, also reside in the large granule fraction.<sup>9, 13, 14, 15</sup>

The microsomes, on the other hand, are submicroscopic elements which have been shown to constitute the chromophilic component of the cytoplasmic ground substance.<sup>7</sup> Whether these elements possess a limiting membrane, like mitochondria, has not been demonstrated, but they become more hydrated and more finely dispersed when placed in hypotonic salt solutions or in distilled water.<sup>8</sup> In the pellet of centrifugation, the microsomes form a perfectly transparent mass, which may be brown or red in color when obtained from liver and colorless when derived from lymphoid tissues or pancreas.<sup>8</sup> The microsomes are complex structures, the major constituents detected, so far, being phospholipids, proteins, and ribose nucleic acid. In contrast with the large granules, no enzymatic functions have been found associated exclusively with the microsomes, although these cell constituents have been shown to possess high thromboplastic activity.<sup>16</sup>

The supernate is perhaps less characteristic than the two other cytoplasmic fractions. It contains proteins, "soluble" enzymes, some ribose nucleic acid, nucleotides, and organic and inorganic compounds of small molecular weight. It is not certain to what extent some of these constituents may have derived from mechanical or enzymatic breakdown of other cell elements during preparation of the extract.

As already pointed out, a separation of the major morphological constituents of the cell can be accomplished by means of centrifugation because of the pronounced differences in sizes which exist between the various groups or families of cellular elements. Whole cells in suspensions can be separated readily from the free cell components, since the next in size, the nucleus, represents no more than 6 to 10 per cent of the volume of the cell. Chromosomes are significantly smaller than nuclei and, in turn, are considerably larger than mitochondria or secretory granules. Similarly, a large gap exists between mitochondria and microsomes and, again, between microsomes and the elements of the supernate.

Similar segregation of the cytoplasmic components into three fractions resembling those just described has been obtained by submitting various types of tissues, namely, liver, pancreas, spleen, lung, mammary gland, embryos, and a number of tumors, to differential centrifugation. It should not be inferred, however, that each of these fractions, especially the large granules and the microsomes, are represented by a single kind of element.

*Complexity of the Main Cytoplasmic Fractions.* Evidence exists which indicates that the large granule fraction, at least, is composed of a variety of elements which are different in constitution and, especially, in functions.

Prominent components of the large granule fraction are the mitochondria. These elements are generally recognized because of their shape, often filamentous or rod-like, and their location in the cell, and for certain staining reactions which, however, are not entirely specific. In embryo, and in undifferentiated tumor cells, mitochondria appear to represent the major part of the large granules which are detectable in the cytoplasm by ordinary

microscopical methods. The large granule fraction derived from these cells, therefore, can be considered to be composed essentially of mitochondria.<sup>20</sup>

The situation is obviously different in cells and organs where several types of large granules are detected microscopically in the cytoplasm and where the cells are known to be engaged in a variety of highly specialized functions. This is the case for the pancreas, where zymogen granules and mitochondria are found in about equal proportions. The case of the liver is somewhat more complex. It has been shown that liver cells contain at least two types of granules, *i.e.*, mitochondria together with secretory granules which are expelled at the time of feeding, a situation very similar to that found in the pancreas.<sup>8</sup> It may be assumed, however, that the liver granules are even more diverse, and it is possible that each of the various liver functions is segregated in different, specialized elements. The problem would be further complicated if, as it has been suggested, secretory granules derive from the progressive transformation of mitochondria or are produced by them.

A separation of granules of nearly the same size is possible when these happen to differ markedly in specific gravity. Thus, the dense melanin granules found in amphibian liver could be separated readily from mitochondria although they are approximately of the same size and shape.<sup>5</sup> Recently, it has been possible to fractionate by centrifugation the large granules of rat liver into "heavy" and "light" portions, the former exhibiting about twice the succinoxidase activity of the latter.<sup>17</sup>

*Fractionation of Large Granules.* The method of differential centrifugation can be applied not only to the separation of various cell components but also to the fractionation of some of the cell components so isolated.

As already noted, the large granules can be separated and washed repeatedly in saline. When placed in distilled water, these elements swell, in the manner of red cells under similar conditions, and finally disintegrate. By means of high-speed centrifugation, it is possible to separate from the suspension a particulate component of submicroscopic size. This small component of large granules has been found to have associated with it most of the ribose nucleic acid originally present in the large granules.<sup>8</sup> The supernate from this high-speed centrifugation was found to contain proteins (among them, undoubtedly, enzymes), ribose nucleotides, and flavins. These two groups of substances of widely different molecular weights can be separated by dialysis.<sup>8</sup> In this manner, some of the morphological features of the large granules were revealed. The presence of small elements, approximately  $0.1\ \mu$  in diameter, detected in mitochondria by electron microscopy,<sup>10</sup> seems to confirm the observations just mentioned.

*Isolation of Chromatin Threads.* The isolation of chromatin threads from the resting nucleus was first reported in December, 1941.<sup>5</sup> The elements which had been separated were stained specifically with the Feulgen technique and, on analysis, were found to contain as much as 40 per cent of desoxyribose nucleic acid. These findings, together with the frequent occurrence of double strands and beaded structures, suggested that the elements isolated represented chromosomes or were derived from them.<sup>6</sup> This method for the mechanical isolation of chromosomal material has been

adopted by Mirsky and his associates and used extensively since then to extract nucleoproteins and study the chemical composition of chromosomes.<sup>18</sup>

*Distribution of Cell Functions.* Microscopical studies, and especially the isolation and *in vitro* analysis of various cell components, have provided conclusive evidence that a cell is not a continuum but is composed of a number of units which are distinct in sizes, chemical composition, and functions. It is the sum of the activities and interactions of these various entities which accounts for the life of the cell. The concept that certain morphological constituents of the cell, such as mitochondria, are reversible systems which could disappear or be created *de novo* in the cytoplasm (coacervates) does not seem to be supported by the recent findings. For example, desoxyribose nucleic acid is found in abundance in chromosomes<sup>6</sup> but, so far, has not been detected anywhere else in the cell. Extensive studies have shown that these bodies of unique chemical composition are the site of segregation of genetic characters. The mechanism by which chromosomes, through individual genes, act to influence the constitution and activities of the rest of the cell is not yet understood. It can be hoped, however, that the possibility of isolating chromosomes, and their analysis *in vitro*, will aid effectively in solving this basic problem of cytology.

Mitochondria appear as the most conspicuous constituents of the cell, next to the nucleus and chromosomes, because of their abundance and typical morphology and behavior. Mitochondria are universally found in active cells, and there is no doubt that they constitute one of the basic building stones of protoplasm. Filamentous or rod-shaped, they are, as a rule, of remarkably constant and uniform width. They are probably self-duplication elements. Unlike chromosomes, however, they do not divide lengthwise but appear to grow by elongation, dividing across their length. Passive division of mitochondria can be clearly observed during mitosis of certain cells, such as the germ cells of the grasshopper and the Arizona *Triton*.<sup>19</sup> Mitochondria have been found to represent an important portion, in dry weight as much as 15 to 20 per cent, of the cell mass.<sup>20, 8</sup> Chemically, mitochondria are characterized by the presence of phospholipids, which probably enter in the constitution of the limiting membrane, and they are the site of important enzymatic activities.<sup>9</sup> As already stated, d-amino acid oxidase activity has been found to be associated with the so-called large granules but not with the other cytoplasmic fractions. It has been found that d-amino acid oxidase activity could be progressively reduced by repeated washing of the granules and restored by the addition of co-factors in the form of heated yeast extract. These results indicate that the two components of the enzyme are both present in the large granules, although one of them is slowly diffusible.<sup>21</sup> Similarly, investigations during the past few years in different laboratories<sup>13, 14</sup> have provided conclusive evidence demonstrating that important members of the respiratory system, namely, cytochrome oxidase and succinoxidase, are located entirely in the large granules. Recent experiments have shown, further, that cytochrome c, a readily soluble component of the system, is also found in the large granules and can be released from them under certain conditions.<sup>15</sup> These observations indicate that the power of respiration is not diffusely distributed throughout the

cell but is probably segregated, together with other enzymatic function, in the formed elements referred to under the general term, large granules. Mitochondria, and the large granules generally, can then be visualized as representing the actual power plants of the cell, where the energy of molecular oxygen is ultimately transferred and utilized. Since it is probable that some of the synthetic activities of the cell take place near the source of energy, it may be assumed that the large granules also represent important manufacturing centers. The findings would support the view, frequently held, that secretory granules are but transformed mitochondria, or are parts of mitochondria, loaded with the accumulated products of their metabolic activity.

In a series of papers, Caspersson and Brachet have developed the view that ribose nucleic acid is related in some way to protein synthesis.<sup>22</sup> This opinion was based, first, on the observation that ribose nucleic acid is always present in the cytoplasm of metabolically active cells and, second, on the fact that ribose nucleic acid is found in greatest abundance in actively growing cells such as embryonic and tumor cells, where synthesis of proteins is presumably needed, and in cells primarily engaged in the production and excretion of proteins, as in the pancreas. The ribose nucleic acid detected by the ultraviolet method or by staining appears to occur in a diffuse form in the cytoplasm (ergastoplasm). On the other hand, observations from this laboratory have shown that most of the ribose nucleic acid of the cytoplasmic ground substance occurs in association with particulate elements of sub-microscopic size, the microsomes.<sup>7</sup> If the ribose nucleic acid of the microsomes is to play a role in protein synthesis, it must be without the immediate participation of the cytochrome-linked respiratory system, since cytochrome oxidase, succinoxidase, and probably cytochrome c are absent. On the other hand, the role attributed to ribose nucleic acid as a factor in protein synthesis is not the only hypothesis that could be held to account for the abundance of this substance in embryonic and tumor cells, since its presence coincides with other outstanding functions of these cells. It may be noted that, in general, the cells which have been found to have a high ribose nucleic acid content, such as embryonic and tumor cells, have been shown by Warburg and his followers to possess to a high degree the power of anaerobic glycolysis. It might be suggested, therefore, that the presence of ribose nucleic acid in cells is related not directly to protein synthesis, but to their capacity for anaerobic respiration. This view would seem to be supported by the concurrence of large amounts of ribose nucleic acid, and of active fermentative processes, in yeasts and in certain bacteria.

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# THE LOCALIZATION AND THE ROLE OF RIBONUCLEIC ACID IN THE CELL

By Jean Brachet

*University of Brussels, Belgium, and University of Pennsylvania, Philadelphia*

**Methods for the Cytochemical Detection of Ribonucleic Acid.** The chemical properties of ribonucleic acid, so far as cytochemical detection and micro-estimation are concerned, depend on: (a) orthophosphoric acid, (b) *d*-ribose, an ordinary pentose, and (c) purine and pyrimidine bases.

The presence of phosphoric acid in nucleic acid molecules confers on them a strong affinity for basic dyes. As a rule, basophilic structures in the cell contain either ribo- or desoxyribonucleic acid. Glycoproteins, also strongly acidic, are usually easy to recognize because of their metachromatic properties.

Ribo- and desoxyribonucleic acids can be distinguished by the fact that only the latter gives a positive Feulgen test, a reaction due to the presence of *d*-2-desoxyribose. Furthermore, nucleases which attack either ribo- or desoxyribonucleic acids are easy to prepare. A useful method for the detection of ribonucleic acid is to treat microscopic sections with ribonuclease. Structures which contained ribonucleic acid before the enzymatic digestion fail to stain with a basic dye, such as toluidine blue, after this treatment. Similar digestion with desoxyribonuclease (thymonucleodepolymerase) results in a negative Feulgen test, as well as the disappearance of basophilia in the chromatin. An interesting combination of basic dyes is Unna's methyl green-pyronine mixture which, as a rule, stains ribonucleic acid-containing elements red and desoxyribonucleic acid-containing elements green. After digestion with ribonuclease, the sections stain only green.

The value of the action of nucleases as a test is, of course, limited by the specificity of the enzymes used. There have been various reports (Schultz,<sup>36</sup> Mazia,<sup>31</sup> Cohen,<sup>21</sup> and Gersh and Bodian<sup>26</sup>) that crystalline ribonuclease has a definite proteolytic activity, which, however, varies strongly from one sample to another (Schneider<sup>35</sup>). According to Baker and Sanders,<sup>1</sup> heating the ribonuclease in a slightly alkaline medium entirely destroys the proteolytic activity without harming the ribonuclease activity. Furthermore, we recently found (Brachet and Shaver<sup>34</sup>) that a crystalline ribonuclease preparation, kindly supplied by Dr. M. Kunitz, had no observable proteolytic activity when sections were treated with the enzyme. After treatment with the ribonuclease, the sections showed no appreciable decrease in the intensity of arginine and tyrosine when subjected to the tests recently described by Serra.<sup>37</sup> These cytochemical tests for amino acids, as used in these experiments, provide a simple method to check the actual proteolytic activity of a given preparation of ribonuclease, in conditions identical to those prevailing for the ribonuclease test itself.

The presence of ribose in the ribonucleic acid molecule cannot, so far, be used for the cytochemical detection of this acid, with the possible exception of Turchini's<sup>41</sup> method (staining of the hydrolyzed sections with a fluorine

derivative), which was reported to give a violet color with desoxyribonucleic acid and an orange color with ribonucleic acid. The transformation of ribose into furfural by acid hydrolysis affords convenient and sensitive methods for the micro-estimation of ribonucleic acid. These methods are valuable, since they often give an opportunity to check the cytochemical observations.

Finally, the strong selective absorption in the ultra-violet band around 2600 Å is the basis of the very fine method of Caspersson<sup>11, 12</sup> for the detection of nucleic acids. Ribose and desoxyribose nucleic acids have nearly identical absorption curves. If both have the same localization in the cell, Caspersson's methods cannot be used to differentiate between them. The combined use of basic dyes, ultra-violet absorption, and the nuclease tests constitutes the best cytochemical method for the detection of nucleic acid now available (Gersh and Bodian<sup>26</sup> and Davidson and Waymouth<sup>29</sup>).

*The Localization of Ribonucleic Acid in the Cell.* Results obtained independently by the ultra-violet absorption method (Caspersson<sup>11, 12</sup>) and by the ribonuclease technique (Brachet<sup>7, 8</sup>) agree perfectly in showing that ribonucleic acid is present in the nucleolus and in the basophilic parts of the cytoplasm, the so-called ergastoplasm. Staining with Unna's mixture combined with ribonuclease digestion indicates, furthermore, the presence of small amounts of ribonucleic acid in chromatin, especially the heterochromatin. This result was corroborated by analysis of isolated nucleic, where 10 per cent of the total nucleic acid was in the form of ribonucleic acid (Brachet<sup>7, 8</sup>).

The amount of basophilic, ultra-violet absorbing material varies considerably from one type of cell to another. Little of it is present in physiologically active organs such as heart, kidney, and striated or smooth muscles. On the other hand, large amounts of ribonucleic acid are present in the exocrine pancreas, the pepsin-secreting cells of the gastric mucosa (excluding the chloride-secreting cells), actively dividing cells from the adult or from the embryo, growing oöcytes, and neurones.

The distribution of ribonucleic acid in various cells and tissues has been further confirmed by micro-chemical quantitative estimation of the ribonucleic acid content of various organs (Brachet<sup>8</sup> and Davidson and Waymouth<sup>29</sup>), which can be arranged in a series, parallel to the basophilia, as follows: pancreas > intestinal and gastric mucosa > liver > spleen > lymph nodes, testis > kidney, muscle, heart, lung. The strongly basophilic young oöcytes of the frog contained about three times more ribonucleic acid per mg. than the large weakly-staining ones. The same relationship was seen on comparing the gastric mucosa with the non-basophilic muscularis.

The peculiar distribution of ribonucleic acid suggests strongly, as pointed out by Caspersson<sup>11, 12</sup> and by myself,<sup>6, 7</sup> that some link must exist between this acid and protein synthesis. The pancreas and the gastric and intestinal mucosa secrete large amounts of enzymatic proteins, while the growing oöcyte is, of course, the site of a tremendous elaboration of proteic yolk. Dividing cells must synthesize their own substances. As for the neurones, it has been shown recently by Weiss<sup>42</sup> that the nerve fibers are the site of a continuous proximo-distal growth and that the protoplasm surrounding the

nucleus of the neurones is continually synthesizing itself. Further evidence for the view that ribonucleic acid is concerned with protein synthesis lies in the fact that the cells in the silk gland of the silkworm caterpillar are, to the best of my knowledge, the richest in ribonucleic acid. The only known function of these cells is the secretion of the protein, silk.

Furthermore, it is interesting to note that changes in the ribonucleic acid content of certain cells have been observed in experimental circumstances which are known to affect protein synthesis. For instance, stimulation of pituitary activity by the injection of estrone (Desclins<sup>24</sup>) or pregnancy hormones (Herlant<sup>28</sup>) led to an increase in the ribonucleic acid content of the gland. By contrast, the prolonged starvation in rats produces a decrease in the ribonucleic acid content of the liver just at the time when it is known that protein synthesis by the liver is no longer possible (Brachet, Jeener, Rosseel, and Thonet<sup>10</sup>). Comparable observations have been reported by Caspersson.<sup>11, 12</sup>

When silkworms contract a virus infection, the so-called jaundice disease, the virus accumulates in the nuclei of the fat body cells in the form of polyhedrons. The first reaction to the infection is an increase in the perinuclear basophilia, together with a striking increase in cell size. The virus thus induces a ribonucleic acid synthesis in the infected cells, followed shortly by growth and protein synthesis (Gratia, Brachet, and Jeener<sup>27</sup>).

It is worth adding that the cytoplasm of cells in the actual process of mitosis usually is much less basophilic than that of adjoining, non-dividing cells. Such observations agree with our previous observation<sup>6</sup> on nucleic acid synthesis in sea-urchin eggs, where quantitative estimation of both types of nucleic acids strongly supports the view that ribonucleic acid can be transformed into desoxyribonucleic acid. Such a transformation is, according to Mitchell,<sup>32</sup> prevented by X radiation of dividing cells, which leads to an accumulation of ribonucleic acid in the cytoplasm. It should, however, be pointed out that, during embryonic development, two antagonistic processes are going on almost simultaneously. One of them is a decrease of the ribonucleic acid content of individual cells at the time of division. The other is the production of the same acid when new proteins are synthesized as a result of organogenesis, differentiation, and growth. Close links between the synthesis of ribonucleic acid and protein during development have been observed in the embryos both of Amphibia (Brachet<sup>7, 8</sup>) and of the chick (Caspersson and Thorell<sup>14</sup>).

*The Constitution of the Cytoplasmic Ribonucleoprotein Granules.* While cytochemical observations strongly suggest a link between ribonucleic acid and protein synthesis, there is no available biochemical evidence in favor of this view. We have, as yet, no indication of the mechanisms by which ribonucleic acid might be an agent in the synthesis of proteins.

Since Claude's<sup>17</sup> work indicated, as early as 1939, that ribonucleic acid is a constituent of cytoplasmic granules which can be isolated by high speed centrifugation, it became important to know more about the chemical composition and the role of these particles. In most of the work which was carried on at the University of Brussels by Jeener, Chantrenne, and myself, no attempt was made, owing to war difficulties and lack of equipment, to

separate the granules into various fractions. The tissues were crushed with sand and extracted with 10 volumes of M/200 phosphate buffer at pH 7.3, and centrifuged at low speed (1500 G.) for 10 minutes. The supernatant fluid was ultracentrifuged for 10 to 20 minutes at 100,000 G. in an air-driven ultracentrifuge. The pellet, which contained both *large granule* and *microsome* fractions (Claude<sup>18, 19, 20</sup>), was used for comparison with the supernatant fluid from the ultracentrifugation. In more recent work by Chantrenne,<sup>16</sup> which will be described later, several fractions, instead of two, have been isolated by differential centrifugation at various speeds.

The granules, a mixture of Claude's<sup>20</sup> mitochondrial and microsomal fractions, could be isolated without difficulty from all vertebrate or invertebrate tissues studied. In all instances, they showed similarities in their chemical constitution, since they always gave positive tests for the following substances: indophenol oxidase, peroxidase, -SH groups, plasmalogen, and ribonucleic acid. The relative intensity of these tests varied from one organ to another, however, and quantitative estimations confirmed this impression. For example, the granules isolated from the pancreas were exceptionally rich in ribonucleic acid, while those from the brain and muscle contained an unusually high amount of phospholipids.

The proteins present in the supernatant fluid after ultracentrifugation gave only weak or negative tests for respiratory enzymes, plasmalogen, and ribonucleic acid. Concerning ribonucleic acid, however, it should be reported that a large portion of this material, which may exceed 50 per cent of the nucleic acid present in the extracts of frog eggs and embryos, as well as of chick embryos, is not sedimented by ultracentrifugation. In the organs of the adult animals, on the other hand, 80 to 90 per cent of the ribonucleic acid extracted from the cells is present in the pellet after ultracentrifugation.

It was already known, from Stern's<sup>39</sup> work, that the particles isolated by ultracentrifugation from heart muscle contained all the cytochrome oxidase, cytochromes, and succinic dehydrogenase which can be extracted with dilute phosphate buffers. The same situation was reported by Chantrenne<sup>18</sup> in yeast cells, where cytochrome oxidase, succinic dehydrogenase, cytochromes a and b, and peroxidase were associated with the granules. Catalase, lactic acid dehydrogenase, carboxylase, and cytochrome c, on the other hand, were present to a much greater extent in the supernatant fluid than in the pellet.

A study of various hydrolases, by Brachet and Jeener,<sup>9</sup> showed that these enzymes are found in the pellet as well as in the supernatant fluid. The enzymes included in this investigation were the following: acid and alkaline phosphatases, ribonuclease, amylase, dipeptidases, cathepsins, trypsin, arginase, and adenylic acid deaminase. Of special interest is the fact that dipeptidases and cathepsins were found in the granules, whatever their origin might have been, and that alkaline phosphatase was so firmly bound to the granules that it could not be removed by repeated washings with buffer solutions. Let us complete the picture by adding that, according to Moog and Steinbach,<sup>33, 40</sup> adenylypyrophosphatase in the chicken embryo is, like the respiratory enzymes of the cytochrome system, present in the granules only.

The next step in our investigation was to find out whether the granules contained the specific proteins elaborated by the various tissues. These studies, due to wartime conditions, were restricted to the pancreas, pituitary, and red blood cells. The results showed that about 60 per cent of the trypsin and at least 20 per cent of the insulin present in the extract of pancreas after low-speed centrifugation was bound to the granules of the pellet. The granules obtained from the hypophysis contained the hormone which produces the expansion of melanophores, while some hemoglobin was always found in the pellet produced from red blood cells. In the latter, it could be shown that this hemoglobin was firmly bound to the granules, since repeated washings failed to remove it.

More recently, Chantrenne<sup>16</sup> has studied the chemical constitution of particles isolated by centrifugation at various speeds. Five different fractions were obtained on centrifugal fractionation of the extract according to the following schedule: Fraction A, 1460 G. for 30 minutes; Fraction B, 5700 G. for 6 minutes; Fraction C, 5700 G. for 60 minutes; Fraction D, 101,000 G. for 6 minutes; and Fraction E, 101,000 G. for 60 minutes. In all five fractions, the content in total nitrogen, ribonucleic acid, lipid phosphorus, alkaline phosphatase, and adenylypyrophosphatase was estimated.

It was found that the smaller particles were relatively richer in ribonucleic acid. The ratio, ribonucleic acid/total nitrogen, increased steadily from Fraction A to Fraction E. In the latter, this ratio was 10 to 15 times larger than in Fraction A. When the content in enzymes and lipid P was related to total nitrogen, it was found that the five fractions, isolated in an arbitrary way, were all different in chemical composition. They cannot possibly be a mixture of only a light and heavy component. This conclusion is supported by observation of the pellets A to E under the ultra-microscope. Each one had its own characteristics; particles in A, B, and C were easy to distinguish, while those in D and E were too small to be seen. These observations led Chantrenne to believe that the granules in the extract were extremely heterogeneous. It is unlikely that they are formed by two entities only, as was assumed by Claude,<sup>20</sup> e.g., by the *large granules* and *microsomes*.

Another interesting point was elucidated by Chantrenne's investigations. If one expresses the results of the analyses in terms of nucleic acid content of the granules, they vary from one fraction to another in a regular way. The larger the particles are, the richer they become in phosphatase, in adenylypyrophosphatase, lipid P, and total nitrogen. As pointed out by Chantrenne, the small granules of Fraction E appear as ribonucleic acid-rich cores around which lipoprotein and enzymes would be synthesized or to which they could attach themselves. Such a hypothesis, in good agreement with Moog and Steinbach's<sup>33, 40</sup> work, is also in agreement with the fact that a large proportion of the ribonucleic acid is not sedimented by high-speed centrifugation of unfertilized frog eggs and early embryos, while a larger proportion can be obtained from tadpole extracts at the same centrifugal speed. It is possible that, during embryogenesis, the small particles become more complex and that this increasing complexity of the granules is an important factor in chemical differentiation.

*Pre-existence of the Granules in the Living Cell—Their Physiological Role.*

It is, of course, of the utmost importance to know whether the granules are already present in the living cell or whether they are only artifacts produced by cytolysis. Careful study of ultracentrifuged pieces of amphibian liver with cytochemical methods brings out strong evidence in favor of the pre-existence, in the living cell, of particles comparable to the granules. After centrifugation of a fragment of frog liver at 40,000 G. for 10 minutes, the basophilic material, which normally is evenly distributed all through the cytoplasm, was thrown down, together with the nucleus, at the centrifugal ends of the cells. These basophilic *crescents* contained ribonucleic acid, as can be readily shown by the ribonuclease test.

We have seen earlier that the isolated granules give positive tests for indophenol oxidase, peroxidase, -SH containing protein, and plasmalogen. All these tests can be used on sections or on small pieces of tissue. It was found that, while they are diffusely positive in the entire cytoplasmic area in the normal liver cells, only the ribonucleoprotein *crescent* stains in the centrifuged liver. It is thus obvious that ribonucleoproteins, -SH containing proteins, plasmalogen, and the respiratory enzymes move together in the ultracentrifuged cell just as they do in the extracts. Since these substances moved towards the centrifugal end of the cell at the same speed used for the isolation of the granules, there is little doubt that they are associated together in the living condition, in a complex identical with or similar to the isolated granules.

This view was further substantiated by an experiment carried out by Chantrenne,<sup>16</sup> at my suggestion, which clearly indicated that cytochrome oxidase was normally bound to granules which play an important role in cellular respiration. Pieces of frog liver were ultracentrifuged at 60,000 G. for four minutes in a solution of saccharose whose density was the same as that of the liver. For purposes of control, non-centrifuged pieces were immersed in the saccharose solution for the same period of time. The oxygen consumption of both fragments was measured and it was seen that ultracentrifugation significantly inhibited respiration.

Further experiments proved that the inhibition involved, essentially, only the cyanide-sensitive respiration which is mediated by cytochrome oxidase. The non-cyanide-sensitive respiratory inhibition obtained by ultracentrifugation was only 6 to 8 per cent, while the cyanide-sensitive respiratory inhibition reached 60 to 70 per cent. These results are similar to those reported by Huff and Boell<sup>17</sup> and by Bodine and Boell<sup>18</sup> for the respiration of *Ascaris* and *Melanoplus* eggs, where ultracentrifugation depressed only the cyanide-sensitive parts of respiration. These observations can be readily explained if one assumes that cytochrome oxidase is bound to granules which ultracentrifugation concentrates in one part of the cell and, accordingly, the cyanide-sensitive respiration in the remaining parts of the cell will necessarily be reduced.

*The Possible Role of the Ribonucleoprotein Granules in Protein Synthesis.*

Any attempt to discuss the role of the ribonucleoprotein granules in protein synthesis is difficult, owing to the scarcity of information on the mechanism of protein synthesis. The most likely possibility (Bergmann and Fruton<sup>2</sup>

and Fruton<sup>25</sup>) is that the action of peptidases and proteases is reversed. The whole problem of protein synthesis by the action of proteolytic enzymes acting on amino acids or peptide mixtures is still in a highly controversial state. There is, however, good evidence, brought forward by Bergmann and his co-workers,<sup>3, 4</sup> that proteolytic enzymes like papain, chymotrypsin, and cathepsin can synthesize a peptide linkage. Such a synthesis, however, is possible only when the synthesized peptides are insoluble. Since the synthesis of peptides is an endo-energetic process, measurable synthesis occurs only when insoluble peptides are formed. Insolubility of the synthesized peptides, of course, displaces the equilibrium of the reaction towards synthesis. It should be pointed out that the physiological dipeptides and tripeptides are not insoluble.

As suggested by Linderstrøm-Lang<sup>30</sup> and by Fruton,<sup>25</sup> it may be that protein synthesis is coupled with exo-energetic processes like oxidation reactions or the breakdown of adenosine triphosphate (ATP) and other compounds that possess energy-rich phosphate bonds.

We cannot, however, entirely dismiss the possibility that other, so far entirely unknown, reactions are responsible for protein synthesis. The situation may be similar to that of glycogen synthesis, which is performed by phosphorylase (Cori<sup>22</sup>), although the action of amylase may also be reversible.

For the time being, we can find out whether the chemical contribution of the nucleoprotein granules agrees in a satisfactory way with the enzyme systems which may conceivably be at work in protein synthesis. It is immediately apparent that such an agreement exists. We always find in the granules the dipeptidases and cathepsin which synthesize peptide linkages under favorable conditions. The granules form a distinct phase in the cell and it is therefore possible that the adsorption of synthesized peptides on such surfaces might shift the equilibria towards synthesis.

Another significant fact is the constant presence in the granules, and only in the granules, of essential respiratory enzymes like cytochrome oxidase and succinic dehydrogenase, as well as of adenylypyrophosphatase. The oxidation reactions and the breakdown of ATP, which have been postulated during protein synthesis, will thus occur in the granules. We saw earlier that the respiratory enzymes, at least the localization in the cell of the granules, is of importance. The fact that the granules always contain some of the specific proteins synthesized by the cells and the progressive complexity of these particles as they become larger constitute additional evidence for their importance in protein synthesis.

The part played by ribonucleic acid itself in protein synthesis is not yet clear. Various suggestions have been made, based mainly on the fact that nucleic acid might combine with the basic groups of amino acids. For example, it is known that nucleic acid combines with many proteins to form insoluble compounds, and it has been suggested by Chantrenne<sup>15, 16</sup> that such a precipitation reaction might be of importance in shifting the equilibrium of the protease reactions towards synthesis.

It seems, however, that a more important lead can be found in Spiegelmann and Kamen's<sup>33</sup> report that there is a flow of phosphorus from the ribo-



nucleoprotein fraction in yeast only when the cells are forced to synthesize proteins. Ribonucleic acid could thus appear as a specific energy donor in protein synthesis as suggested by Spiegelmann and Kamen. It is, of course, a striking fact that ribonucleic acid is among the constant constituents of the granules.

It should, however, be kept in mind that the role of the granules as agents of protein synthesis rests only on circumstantial evidence and not on definite proof. Much more work will be necessary before the exact status of these particles, which have so many characteristics in common with viruses and plasmagenes, can be evaluated.

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*Discussion*

DR. MAX M. FRIEDMAN (*Queens General Hospital, Jamaica, L. I., N. Y.*): In muscle and heart tissue, the ribose nucleotide content is relatively high and the ribose nucleic acid is low, while in the liver, the reverse occurs. Is there any possible significance to this from the consideration of polymerization equilibrium?

DR. BRACHET: The relationship between ribonucleic acid and ribomononucleotides is still far from clear. There are indications from Ostern's work that, in yeast cells, zymonucleic acid might be transformed into muscle adenylic acid, adenosine triphosphate and, eventually, into coenzymes of the dinucleotide type.

# THE SURFACE CHEMICAL PROPERTIES OF CYTOPLASMIC PROTEINS

By M. J. Kopac

Department of Biology, New York University, and Marine Biological Laboratory,  
Woods Hole, Massachusetts

This report deals with the interfacial denaturation, at oil-water interfaces, of proteins within the living cell as well as those obtained by the centrifugal fractionation of rapidly disintegrated cells. Cytoplasmic proteins undergo little, if any, surface denaturation at experimentally introduced oil-water interfaces providing the cytoplasm remains intact. If the cell is cytolyzed within 30 to 60 seconds after the injection of an oil drop into intact cytoplasm, the surface denaturation of the released proteins is so extensive that the entire interfacial area becomes occupied by protein molecules in various stages of unfolding. The rapid adsorption of proteins and subsequent unfolding causes the interfacial area to expand, thereby producing a crinkled interface—the Devaux effect\* (FIGURES 1,A and C, 1,D-G). In the absence of cytolysis, the injected oil drop remains spherical (FIGURE 1,A).

The terms, *surface* or *interfacial denaturation*, as employed in this report, represent the changes in architecture of a globular, 3-dimensional protein molecule to an unfolded, essentially 2-dimensional configuration induced primarily by surface or interfacial forces. The term, *adsorption*, is employed in the classical sense, *i.e.*, concentration of substance at phase boundaries.

The immature eggs of the starfish, *Asterias forbesii*, were employed for the exploratory investigations on the surface chemical properties of cytoplasmic proteins. There were several reasons for this choice of material: (1) the oocytes are large enough so that oil drops can be readily injected into the large germinal vesicle or into the cytoplasm; (2) oil drops may be injected into the cytoplasm without danger of cytolysis, since the cytoplasm of the immature starfish oocyte is remarkably resistant to microsurgical injuries (Chambers<sup>1</sup>); and (3) cytolysis may be readily induced.†

Although the cytoplasm is resistant to injuries produced by careful microinjections of oil drops, the germinal vesicle, by contrast, is extremely susceptible to mechanical injuries. A slight puncture of the germinal vesicle with a microneedle, at room temperatures, is sufficient to initiate rapid cytolysis of the germinal vesicle, nucleolus, and cytoplasm (Chambers<sup>1</sup>).

Thus, one or several oil drops may be safely injected into the cytoplasm. At any given instant, cytolysis may be induced by stabbing the germinal vesicle with a microneedle. The usual micromanipulative procedures as described by Chambers and Kopac<sup>2</sup> were employed.

\* Preliminary descriptions of the Devaux effect first appeared in the following: Kopac. Biol. Bull. 71: 398. 1936; Biol. Bull. 75: 351. 1938; Microsurgical and Germ Free Methods, pp. 62-76. Thomas, Springfield, Ill. 1943; Colloid Chemistry, vol. 5. pp. 875-883. Reinhold, New York. 1944; Chambers. AAAA Publ. No 14. pp. 20-30. Science Press. 1940.

† At the conference at which this paper was delivered, a motion picture was presented which showed the microinjection of oil drops into the cytoplasm of *Asterias* oocytes, the induction of cytolysis by puncturing the germinal vesicle, the cytolytic reaction, and subsequent development of the Devaux effect at the oil-water interface.

*Experimental Modification of Spontaneous Devaux Effects**A. Time of Cytolysis in Relation to Injection of Oil (Kopac<sup>3</sup>).*

As shown in FIGURE 1,B, oil drops, after being in contact with intact cytoplasm for several minutes, undergo changes in interfacial properties that prevent the development of the Devaux effect when cytolysis occurs. The effects of various interfacial forces on surface denaturation of proteins will be discussed later.

A series of measurements was made to determine the relation of the time of cytolysis and of the oil injection to the production of Devaux effects. The oil drops were injected at predetermined intervals, either before or after cytolysis. The data are summarized in FIGURE 2.

These data clearly show that rapid, spontaneous Devaux effects developed in nearly every instance when the oils were introduced at or near the onset of cytolysis. If the oil drop remained in contact with cytoplasm for 2+ minutes, about 50 per cent of the drops developed typical Devaux effects. Others crinkled slowly or else the degree of crinkling was moderate (see FIGURES 1,D and 1,E). The critical time for Devaux effect production is therefore about two minutes before cytolysis. Oil drops in contact with cytoplasm for 5+ minutes remained unchanged on cytolysis, as shown in FIGURE 1,B.

On the other hand, if the oils were injected into the cytolized residue (post-cytolytic period), the probability of obtaining rapid and intense Devaux effects decreased the longer the interval between cytolysis and injection of the oil. Although all drops crinkled if injected within 15 seconds after cytolysis, only 50 per cent did so when brought in contact with the residue 30 seconds after cytolysis. The spontaneous crinkling diminished in magnitude until, at about 4 or 5 minutes, no visible surface effects were produced. On occasion, oil drops injected 3 minutes after cytolysis slowly developed a mild crinkling (FIGURE 1,D).

The surface denaturation of cytoplasmic proteins during the post-cytolytic period will be further discussed in the section on drop-retraction technique.

*B. Effects of Immersion Media on the Production of Devaux Effects.*

The *Asterias* oocytes were immersed in various media for periods ranging from 30 minutes to two hours. The percentages of oil drops that developed spontaneous Devaux effects within 45 seconds after cytolysis are given in TABLE 1 (Kopac<sup>4</sup>).

Oocytes immersed in sea water did not always yield the typical Devaux effect if the oil drops were injected two or more minutes before cytolysis (FIGURE 2). On the other hand, oocytes immersed in NaCl or KCl solutions produced spontaneous Devaux effects on oil drops injected up to 4 minutes before cytolysis. NaCl or KCl solutions, obviously, did not produce the same changes at the oil-cytoplasm interface as those observed in oocytes immersed in sea water. The surface denaturation of proteins at oil-water interfaces, as will be discussed later, depends to a considerable degree on the characteristics of the interface.

The action of urea was unexpected. At lower concentrations (0.3 to 0.5M), urea inhibited the production of spontaneous Devaux effects during

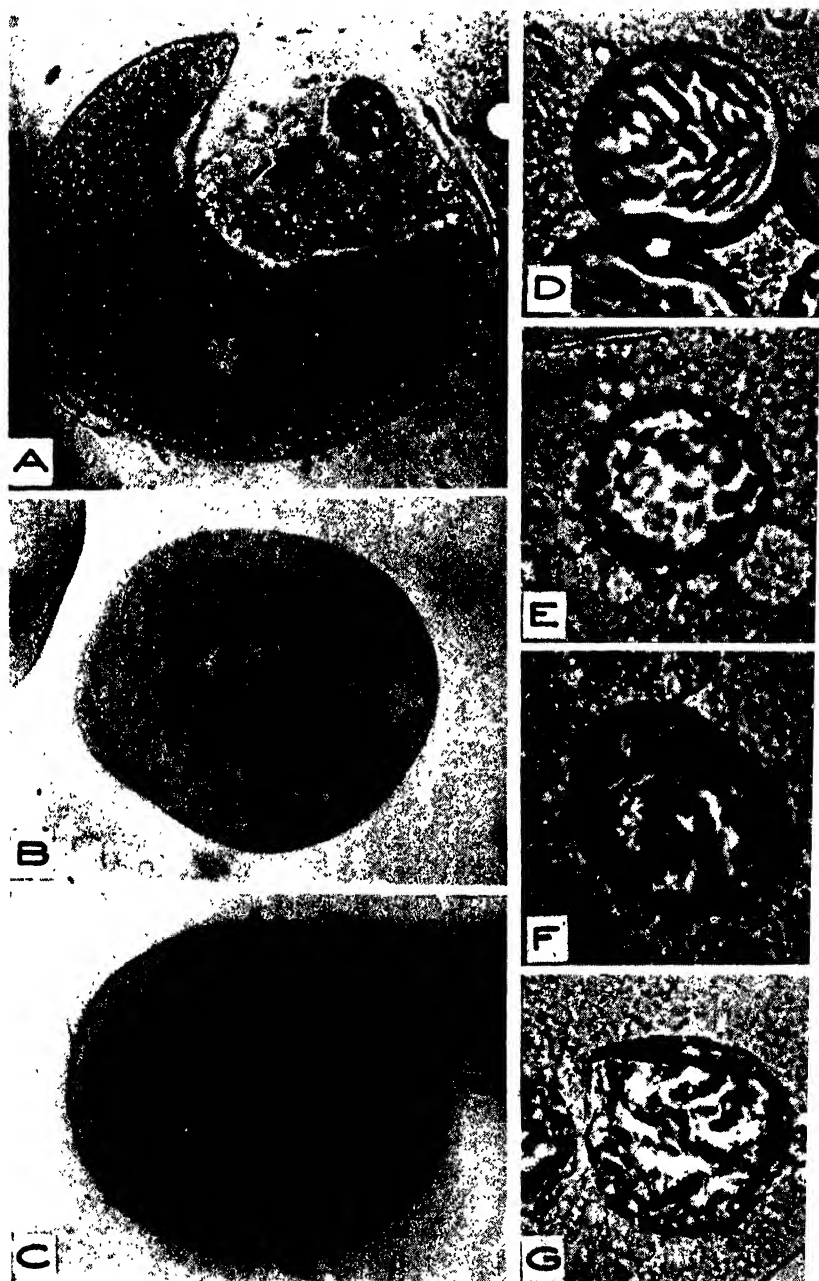


FIGURE 1, A-G. (For description see facing page.)

the post-cytolytic periods, although the inhibition on oil drops injected before cytolysis was negligible. At the higher concentration (0.67M), the production of Devaux effects was completely inhibited no matter when the oil was injected into the oocytes. Not only was the Devaux effect suppressed if the oil drops were injected at the instant of cytolysis, normally the optimum time, but there was no suggestion of even mild crinkling. Such oil drops remained permanently spherical.

*C. Cytoplasmic Cytolysis Induced by Rapid Microinjection of Aqueous Solutions.*

After injecting a drop of oil into the cytoplasm of an *Asterias* oocyte, a second micropipette was employed to inject rapidly a small volume of aqueous solution into the cytoplasm adjacent to the oil drop. Under such conditions, the injection produced a localized cytolytic zone surrounding the oil drop as shown in FIGURE 1,C.

*Injections of Salt-free Solutions + Phenol Red.* Injections of small volumes of salt-free solutions of phenol red (0.04 per cent) frequently produced small cytolytic zones around only a portion of the oil drop. Accordingly, a portion of the oil drop remained in contact with cytoplasm. The surface of the oil drop, in contact with the cytolytic residue, did not crinkle. The surface, originally in contact with cytoplasm, however, produced a typical Devaux effect after the remaining cytoplasm became cytolized.

The phenol red changed in color from red to yellow in the cytolytic zone. Within 30 seconds, the red color reappeared, indicating the infiltration of sea water into the injured zone. Whenever the phenol red in the cytolytic zone remained yellow, no Devaux effects were produced at the oil-water interface.

If a pre-cytolytic zone (narrow zone of cytolytic residue surrounding the oil drop) existed prior to the injection of phenol red, the new cytolytic zone remained red and, in such instances, a mild Devaux effect was developed. This might indicate that the pre-cytolytic zone resulted from the injection of small volumes of sea water simultaneously with the oil. If a pre-cytolytic

FIGURE 1, A-G (See opposite page). Photographs of oil drops microinjected into *Asterias* oocytes and the effects of cytolysis.

A. Partially cytolized *Asterias* oocyte. *Asterias* oocyte showing an oil drop (Percomorph oil) injected into intact cytoplasm. Note the sphericity and clear-cut contour of oil drop. Another oil drop was injected into germinal vesicle which produced immediate cytolysis. Note the irregular shape and contours of oil mass in cytolytic residue. The change in shape of the oil drop, from a sphere to an irregular mass, occurred in less than 30 seconds. This reaction is called the spontaneous Devaux effect.

B. Oil drops in a cytolized *Asterias* oocyte. The first drop was injected into an intact *Asterias* oocyte 8 minutes before cytolysis, the second, one minute before cytolysis. Immediately after cytolysis, the second drop formed a spontaneous Devaux effect within 45 seconds. The first drop remained spherical and did not develop a Devaux effect. Photograph was taken 30 minutes after cytolysis of oocyte.

C. *Asterias* oocyte with small cytolytic zone surrounding oil drop. An oil drop was injected into intact cytoplasm of an *Asterias* oocyte. If small volumes of aqueous substances are rapidly microinjected, localized cytolysis may be induced. Note the clear-cut Devaux effect that has developed at the oil-water interface. Equilibrium shapes of oil drops following cytolysis of *Asterias* oocytes.

D. The introduction of an oil drop into the cytolytic residue several minutes after cytolysis rarely produces a typical spontaneous Devaux effect. In most instances, only a few crinkles appear at the interface, and these can be demonstrated only if the drop is slightly distorted, for example, by pressing against another oil drop. Note the clear-cut contour of the drop and the spherical shape. Oil drops introduced 5 minutes before cytolysis rarely show more extensive crinkling.

E. Oil drops introduced 1.5 to 2 minutes before cytolysis generally produce a spontaneous Devaux effect of the type illustrated by this photograph. The crinkling is extensive, but the general shape of the drop is spherical.

F. More extensive crinkling produces a considerable distortion in shape of the oil drop.

G. Introduction of appropriate oil-water interfaces at the instant of cytolysis produces the remarkably crinkled and distorted drop shown in this photograph. This is a typical Devaux effect, and the time for maximum crinkling rarely requires more than 30 seconds.

zone was not formed, the Devaux effect failed to appear following the cytolysis induced by phenol red injections.

It is evident that salt-free solutions inhibited the formation of the Devaux effect, but this might be modified either by an alkaline reaction of the cytolytic zone, the entry of sea water, or by the presence of a pre-cytolytic zone.

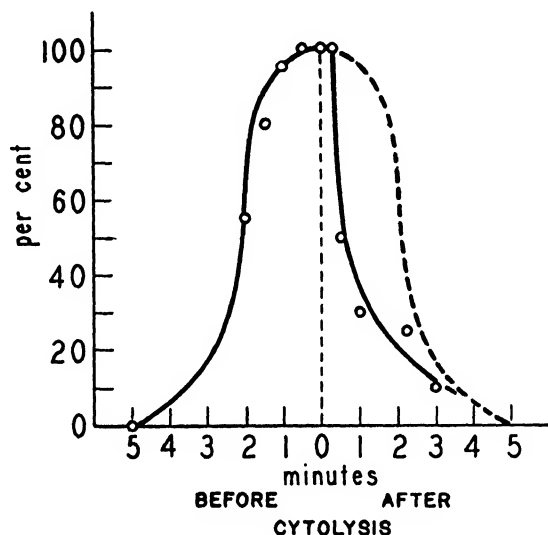


FIGURE 2. The production of spontaneous Devaux effects in relation to time of cytolysis and injection of oil.

The x-axis gives the time in minutes at which the drops were injected either before or after cytolysis. The y-axis gives the percentage of oil drops, at the various injection times, that developed spontaneous Devaux effects within 45 seconds. The extent of crinkling equalled or exceeded that shown by FIGURE 1E.

TABLE 1

Immersion media	Time of injecting oil in relation to cytolysis	
	1 minute before	1 minute after
Sea water	100 per cent	30 per cent
NaCl 0.52M	90	20
KCl 0.53M		
CaCl <sub>2</sub> 0.34M		
MgCl <sub>2</sub> 0.37M		
Sea water + urea (0.33 to 0.5M)	90	0
Sea water + urea (0.67M)	0	0

*Injections of Potassium Chloride Solutions (0.53M).* The oil drops usually developed a moderate Devaux effect (FIGURE 1,E) following cytolysis induced by injections of KCl solutions. In a few isolated instances, typical Devaux effects (FIGURES 1,F and 1,G) were obtained within 30 to 60 seconds.

If a pre-cytolytic zone existed around the oil drop, such residue remained attached to the oil drop following injection of the KCl solution. The residue was hyaline at first, then it quickly disintegrated to a granular residue.

Thus, the KCl solutions had a tendency to inhibit not only the rate but also the intensity of the crinkling reaction at the surfaces of oil drops.

*Injections of Calcium Chloride Solutions (0.34M).* Spontaneous Devaux effects were produced within 30 to 60 seconds after introduction of the calcium chloride solution. The Devaux effects obtained after cytolysis induced by calcium chloride injections were of the same magnitude regardless of the presence or absence of a pre-cytolytic residue which was usually coagulated. If a pre-cytolytic zone existed, the action of the calcium chloride was to form a shell of coagulated residue around the oil drop.

Oil drops were frequently surrounded by granules following cytolysis by calcium chloride. In most instances, these disappeared and the Devaux effect developed normally. There was no evidence to indicate that these granules were responsible for the Devaux effect. The action of calcium chloride on the Devaux effect was not striking; however, there is a suggestion that the production of the effect was slightly enhanced.

*D. Action of Strong Protein Precipitating Agents (Kopac<sup>5</sup>).*

The injection of trichloroacetic acid into intact *Asterias* oocytes immediately fixed all structures. Within a few seconds, the trichloroacetate-treated eggs became so hard that oil could not be injected into them. Injection of trichloroacetate into cytolysed eggs produced an immediate coagulation of the cytolytic residue.

Drops of oil were injected into the cytoplasm of intact oocytes and cytolysis was induced by puncturing the germinal vesicle with a microneedle.

If trichloroacetate was injected into the cytolytic residue before the Devaux effect appeared, no further changes at the interface appeared, since the sphericity and smooth contours of the drops were preserved. If trichloroacetate was injected at a time when the oil drops were crinkling, no further increase in the Devaux effect occurred.

In every instance, when the precipitant was added after the oil drops had developed the Devaux effect, a reduction occurred in the extent and number of crinkles, *e.g.*, the return to a condition of minimum surface area. Thus, oil drops that were crinkled as shown in FIGURE 1,F before trichloroacetate was added generally returned to a shape shown in FIGURE 1,D. These attempts to regain a spherical shape may be due to the shrinking action of the reagent on the adsorbed and on adjacent proteins of the cytolytic residue.

The action of trichloroacetate may be summarized as follows:

Oil + cytolysis.....	Devaux effect [progressive development].
Oil + cytolysis + trichloroacetate....	Stops development of Devaux effect.
Devaux effect + trichloroacetate .....	Reversal, to spherical shape.

The injection of an aqueous solution of phosphotungstic acid into cytolysed oocytes produced an immediate coagulation. Frequently, on cytolysis there may be an outflow of cytolytic residue through a torn portion of the vitelline membrane (FIGURE 9,A). Injection of phosphotungstate into such oocytes immediately stopped the outflow of such cytolytic residue. The action of the reagent was localized, and small amounts of it produced only small coagulation zones.



With oil drops injected into intact oocytes and cytolysis produced in the usual manner, the effects of phosphotungstate were to stop further development of the Devaux effect. As soon as the precipitant came in contact with the region surrounding the oil drop, no further changes occurred either in increasing the number of folds or in decreasing the extent of crinkling (see trichloracetate effect). Even after prolonged standing (12 to 24 hours), there was no tendency of the distorted drops to return to spherical shapes.

Accordingly, the action of phosphotungstate may be summarized as follows:

Oil + cytolysis . . . . .	Devaux effect [progressive development].
Oil + cytolysis + phosphotungstate . . . . .	Stops further development of Devaux effect.
Devaux effect + phosphotungstate . . . . .	No reversal.

To summarize, the action of either trichloracetate or of phosphotungstate was to prevent further development of the Devaux effect. Trichloracetate reduced the interfacial area of the crinkled drop, while phosphotungstate left the crinkled interface unchanged. In both instances, the most striking effect was the immediate stoppage of any further increase in crinkling, folding, or distortion of the oil drops. When the precipitants were added before crinkling had started, such drops retained their original sphericity and smooth contours. In other words, these reagents inhibited the surface denaturation of cytoplasmic proteins.

#### *E. Devaux Effect in Centrifuged Oocytes and Mature Eggs (Kopac<sup>6</sup>).*

Centrifugal technique was employed for separating the formed components within the cells. *Asterias* eggs, immature or mature, may be centrifuged for 10 to 12 minutes at 6 to 7 kilogravities without serious injury, providing the cells are suspended in an isopycnotic mixture of sucrose and sea water. Following such centrifugation, the formed components of mature eggs were separated into three zones: (1) an oil cap, composed of oil globules, at the extreme centripetal pole; (2) a large granular layer at the centrifugal pole; and (3) a narrow, hyaline band consisting of cytoplasmic matrix, between the oil cap and the granular zone (see also Costello<sup>7</sup>).

A hyaline zone did not appear in the cytoplasm of centrifuged, immature eggs, since the centripetal pole was occupied by the persisting germinal vesicle. It is of interest to note that the nucleolus is the densest structure within the germinal vesicle, since its location after centrifugation is always in the extreme centrifugal zone of the vesicle.

*Hyaline versus Granular Zones in Centrifuged Eggs.* Oil drops were injected into the hyaline and into the granular zones of centrifuged, mature eggs. The cell was induced to disintegrate by repeated prodding with a microneedle. The rate and extent of development of the Devaux effect was recorded for both oil drops.

The differences in the degree of development of the Devaux effect on the two drops were indeed striking. The oil drop in the centrifugal, granular zone quickly became distorted and covered with numerous folds (FIGURE 1,G), while the oil drop in the hyaline zone either formed a few surface

crinkles (FIGURE 1,D) or remained unchanged. The rates of development were considerably different. The Devaux effect was developed on the oil drop in the granular zone within a few seconds, while in the hyaline zone, if changes occurred, a considerable period of time elapsed before these changes became apparent. In no instance did an oil drop, placed in the hyaline zone, attain the degree of crinkling shown, for example, by FIGURE 1,E.

The typical experiment, following cytolysis, would show a heavily crinkled oil drop near the centrifugal end and a spherical, relatively unchanged oil drop near the centripetal end. The average time for oil drops, placed in the centrifugal, granular zone, to develop a maximum Devaux effect was about 15 seconds, and several instances were noted where such changes occurred in less than 7 seconds.

Another significant point was that complete cytolysis of the granular zone was unnecessary. The oil drop needed only to be surrounded by a small cytolytic zone to produce the striking, spontaneous Devaux effect.

*Centrifuged, Immature Oocytes.* Centrifuged oocytes produced different reactions from those observed in similarly treated, mature eggs. Employing the same procedures as outlined above, an intense and rapid Devaux effect was produced in both the germinal vesicular zone and in the granular zone. The rates and intensities were essentially the same. The average time for complete crinkling (FIGURE 1,G), considering all instances, ranged from 15 to 40 seconds.

Since no visible granules were present in the germinal vesicle, the results obtained with the oil drops are of considerable interest. It is obvious that the hyaline substance within the germinal vesicle differs from the hyaline zone of the centrifuged, mature egg. Thus, by use of the Devaux effect as a criterion, the two hyaline-appearing substances could be as clearly differentiated as by the use of ultra-violet light absorption (see Harvey and Lavin<sup>8</sup> regarding the *Arbacia* oocyte).

In centrifuged, mature eggs, an intense Devaux effect occasionally developed in the hyaline zone. However, this was due to the disintegration of the membrane of the germinal vesicle following centrifugation. In such examples, the contents originally present in the germinal vesicle were still confined to the apparent hyaline zone of the matrix. Thus, if the germinal vesicular membrane was allowed to break down, following centrifugation, before the oil drops were injected, rapid and intense Devaux effects could be demonstrated on subsequent injection of oil drops.

In other instances, although the Devaux effect did occur in the apparent hyaline zone, the effect was slower and less intense (FIGURES 1,E and 1,F) than in the previous examples. Such an effect might have resulted from the disintegration of the membrane during centrifugation and the diffusion of the contents of the germinal vesicle into the surrounding cytoplasm. The inevitable redistribution of cellular inclusions, following centrifugation, produced a dilution of material from the germinal vesicle which is responsible for the Devaux effect in immature eggs. Only in those cells which matured prior to centrifugation was the Devaux effect negligible in the hyaline zone.

It is reasonable to suppose that the substances in the germinal vesicle which become dispersed in the cytoplasm following maturation may be carried by the dense granules during centrifugation. This would tend to deplete the hyaline zone of those substances that promote the development of spontaneous Devaux effects.

*Comparison of the Rates of Devaux Effect Development in Centrifuged and in Uncentrifuged Eggs.* The role of the granular components of the *Asterias* egg in producing the Devaux effect on oil drops was analyzed in another way. A tabulation was made of the times required for complete crinkling on oil drops injected into uncentrifuged, mature eggs or into the granular zones of centrifuged eggs.

The results are summarized in TABLE 2.<sup>5</sup>

The times given in TABLE 2 are the intervals from the instant of cytolysis to completion of the Devaux effect. Only those drops on which crinkling occurred were considered. In the uncentrifuged cells, 4 out of 112 (ca. 4 per cent) failed to reach the stage illustrated by FIGURE 1,G within 45 seconds, but the crinkling was completed within 50 to 60 seconds.

TABLE 2

Per cent of oil drops showing maximum Devaux effect	Time in seconds for development of Devaux effect		
	Centrifuged, granular zone.	Uncentrifuged, cytoplasm.	Uncentrifuged Centrifuged
70	17	30	1.76
80	18	32	1.78
90	20	36	1.80
100	30	45	1.50

The above data show that the granular components are responsible for the increased crinkling rates in mature eggs. The granules are concentrated by centrifugation and, accordingly, the degree of crinkling seems to be a function of the concentration of the adsorbable material.\*

*Disintegration of Granules Stained with Methylene Violet.* *Asterias* eggs readily accumulated the thiazine dye, methylene violet (Bernthsen), from dilute solutions in sea water. The same granules that could be stratified centrifugally were stained blue. Under low power, the uncentrifuged eggs appeared to be uniformly stained, but this was due to the large number and regular distribution of the granules. The nucleolus also became stained, but more slowly than the granules.

Striking changes in coloration of the cytoplasm of uncentrifuged eggs occurred after cytolysis. On cytolysis, the stained granules disintegrated. Where a large cytolytic zone was produced, the cytoplasmic residue became

\* According to Costello,<sup>7</sup> the packed volume of granules in *Asterias* eggs is ca. 77 per cent of the total egg volume. On correcting this volume for packing and subtracting the volume occupied by intergranular matrix, the volume becomes ca. 57 per cent. Thus, on centrifugation, the concentration of the granules becomes 0.74 of the total volume occupied. Originally, these granules occupied 0.57 of the egg volume. The ratio,  $0.74/0.57 = 1.34$ , or a 34 per cent increase in granular concentration. The increase in rates of Devaux effect development on centrifugation ranges from 50 to 80 per cent (TABLE 2). These values agree surprisingly well with those calculated from Costello's data.

faintly colored, while the granules in the intact cytoplasm retained their blue color.

Following the injection of oil drops into the cytoplasm of stained eggs, and on inducing cytolysis only around the oil drop, the effects most frequently observed were the following: A typical spontaneous Devaux effect was developed on the oil drop (FIGURE 1,G). The granules disintegrated in the cytolized zone, which became either colorless or faint blue. In many instances, the surface of the oil drop acquired a blue color. As long as the granules remained intact, the color was retained by them.

Stained, centrifuged eggs gave results that were identical to those obtained with unstained cells. On cytolysis, the stained granules disintegrated and the oil drops became crinkled. Oil drops placed into the hyaline zone behaved similarly to those placed in the hyaline zone of unstained eggs. If the stained granules were present in small amounts, the Devaux effects were moderate (FIGURE 1,E). Maximum effects were obtained where the granules were the most numerous, *i.e.*, in the centrifugal zone.

In stained, immature eggs which were also centrifuged, the Devaux effects were obtained in the germinal vesicular zone even though there was no accumulation of the dye, since the granules were sparse. At best, the concentration of stained granules in the region of the germinal vesicle of strongly centrifuged eggs was low. The differentiation of the germinal vesicular contents from the hyaline cytoplasmic matrix was again demonstrated.

*Disintegration of Granules Stained by Rhodamine.* The granules of *Asterias* eggs were stained a deep pink color while the hyaline matrix was only faintly stained in cells immersed in dilute solutions of rhodamine in sea water.\* On cytolysis, the granules disintegrated as usual, releasing the dye, which in turn stained the non-granular components a deeper pink color. The Devaux effects were identical to those obtained in unstained eggs or in eggs stained with methylene violet.

Even when the cytolysis was confined to the region surrounding the oil drop, the intact cytoplasm as well as the cytolized zone was stained. The latter effect, therefore, is different from that obtained under similar conditions in eggs stained with methylene violet.

It is evident, therefore, that the Devaux effects obtained at oil-water interfaces placed in contact with the cytoplasm of *Asterias* eggs depends on the breakdown of those granules which accumulate methylene violet or Rhodamine. These dyes had no effect on either the rate or extent of Devaux-effect development. The rates and intensities of crinkling at oil-water interfaces, however, were increased in accordance with the concentration of the granules.

The Devaux effects, on the other hand, that were obtained from the germinal vesicle or contents of this structure were independent of granular disintegration. Such effects resulted from the adsorption and subsequent surface denaturation of hyaline components present in the non-granular structure of the germinal vesicle.

\* E. B. Harvey (Biol. Bull. 81: 114. 1941) reported that the yolk granules and mitochondria of *Arbacia* eggs are stained a pink color by rhodamine. The upper portion of the matrix fraction also stained a pink color with rhodamine.

The most significant observations were that Devaux effects could not be obtained unless a significant amount of cytolysis occurred around the oil drop, regardless of the nature of the oil-water interface. While the cytoplasmic or the germinal vesicular structures remained intact, the injected oil drops remained spherical as shown in FIGURE 1,A.

### *The Drop-Retraktion Technique*

The striking differences between cytoplasmic proteins, *in vivo*, and those obtained after cytolysis, as demonstrated by surface chemical behavior, required an investigation of the interfacial denaturation of simpler proteins and the effects of various agents on these properties.

One of the most useful methods to be developed for these determinations has been the drop-retraction technique and the micro-tensiometer (Kopac<sup>9, 10</sup>). These techniques are micro-adaptations of surface chemical procedures, originally developed by Devaux,<sup>11</sup> and extended by Langmuir and Waugh,<sup>12</sup> for the investigations of protein films at oil-water interfaces.

The apparatus permits the measurement of surface denaturation of proteins at oil-water interfaces in which the oil drops are of the same dimensions as those shown in demonstrating spontaneous Devaux effects (FIGURE 1,A to G) and post-cytolytic phenomena (FIGURE 9,A and B).

Several new features have been added to the apparatus and to the procedures since the drop-retraction technique was first described (Kopac<sup>10</sup>). These will be discussed and to this will be added representative data on model protein and nucleoprotein systems.

With the drop-retraction apparatus, the surface area of an oil drop, poised on the tip of a micropipette, can be increased or decreased by enlarging or reducing its diameter. FIGURE 5 is a pictograph illustrating the technique and FIGURE 8 illustrates the apparatus with which the dimensions of an oil drop may be precisely controlled and measured.

The micropipettes were made by hand in such a way that during the pulling-out process an orifice was formed and fire-polished. As described by Barber,<sup>13</sup> pipettes with a small polished opening are obtained when the glass, in a half-molten state, parts with a gentle snap. To produce such pipettes, one must pull suddenly when the glass begins to melt in the micro-flame. The diameters of the orifices ranged from 2.5 to 10 microns. Since the orifice is fire-polished and at right angles to the axis of the pipette, the oil drop remains poised on the tip of the pipette as shown in FIGURE 4,A.

The manual control of the drop-retractor (FIGURE 5) is employed to charge the micropipette with a volume of oil by filling through the tip from a small hanging drop of oil previously placed on a coverslip and mounted on the moist chamber. With the micropipette filled with oil, and the aqueous phase in place, the tip of the micropipette is inserted into the hanging drop. The pressure necessary to expel the oil can be developed by manipulating the micrometer head. If not too large, the spherical drop will remain attached to the microtip as shown in FIGURE 4,A. If too large, it will break away, and the pipette must be refilled with a smaller volume of oil.

Factors which limit the maximum size of an oil drop that will remain

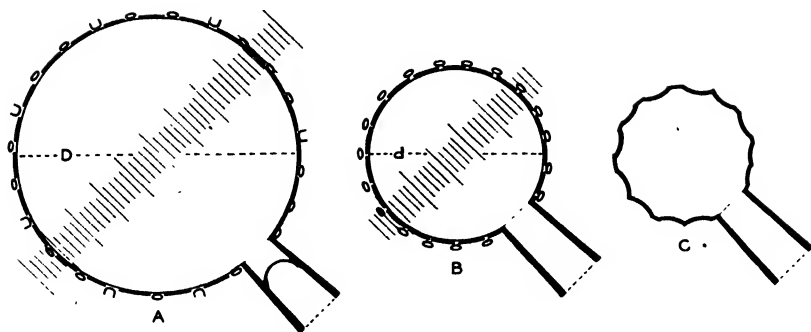


FIGURE 3. Diagrams of oil drops at various stages of retraction. These diagrams, representing cross sections of oil drops attached to the micropipette, explain the crinkling effect.

A. In an aqueous phase containing proteins, an oil-water interface, at any time, will contain protein molecules in various stages of unfolding. First, some molecules remain as 'native,' 3-dimensional structures (ellipsoids). Second, other molecules will be partially unfolded (U-shaped). Third, still others will be completely unfolded (2-dimensional platelets).

B. The interfacial area becomes reduced on retracing the oil drop. Molecules present in the interfacial zone are subjected to increasing surface pressures as the interfacial area is reduced. As the surface pressure is increased, some of the molecules present in A will be squeezed out of the interface. The first molecules to be expelled will be the 3-dimensional molecules since they have undergone no changes in structure and, accordingly, their original solubility in the aqueous phase is preserved. Further decrease in interfacial area with accompanying increase in surface pressure will expel the partially unfolded molecules. The surface pressure needed to force out the partially unfolded molecules may depend on the degree of unfolding. It is possible that partially unfolded molecules cannot be completely expelled, but only the less unfolded portions will be pushed out of the interfacial area. The fully unfolded molecules cannot be driven out of the interface no matter how high the surface pressure. These molecules have oil on one side and water on the other. This figure represents the condition of the interface at the critical diameter,  $d$  (see also FIGURE 6).

C. If the interfacial area is further reduced, crinkling will result, since the interfacial area cannot be made any smaller due to the presence of the nondisplaceable, unfolded molecules. Reduction of the volume of the enclosed mass while interfacial area remains constant produces the crinkling effect.

The diameters,  $D$  and  $d$ , of the oil drops can be measured with a calibrated micrometer ocular. The drops may also be photographed (FIGURES 6 and 8) and the measurements obtained later from negatives.

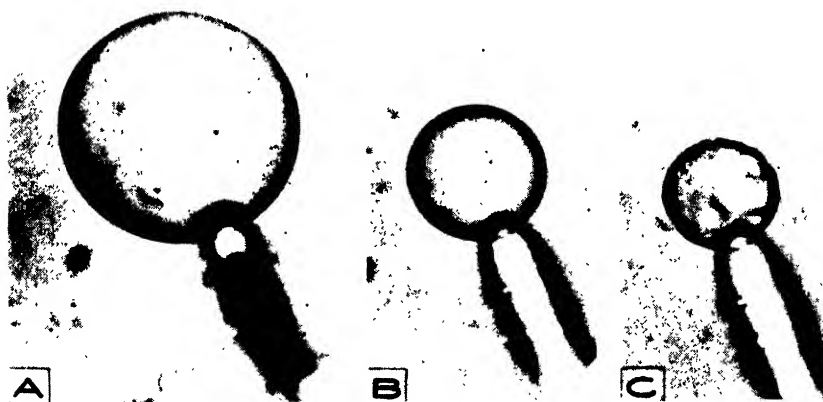


FIGURE 4. Photographs of oil drops in contact with an aqueous phase containing proteins.

Practical application of the drop-retraction method. Note the small volume of oil remaining in the tip of the micropipette. With this oil plug, the oil may be drawn back into the pipette by reducing the back pressure with the drop-retractor (FIGURE 7).

The surface area of the first drop,  $A = \pi D^2$ , where  $D$  is its diameter. With the oil phase immersed in clean water or in inorganic salt solutions, the drop maintains a spherical shape until it is completely retracted. If, however, the oil first comes in contact with an aqueous phase containing proteins, the retraction of the oil produces strikingly different effects. As the drop (B) is retracted, by reducing the back pressure, the sphericity of the drop will be maintained to the critical diameter,  $d$ . Its surface area,  $s = \pi d^2$ .

If the drop (C) is made any smaller, the surface becomes crinkled, thereby simulating the spontaneous Devaux effect.

attached to the micropipette are the following: (1) the weight of the oil drop ( $= V \Delta d g$ ), where  $V$  = volume of oil drop (cc),  $\Delta d$  = difference in density between oil and aqueous phase,  $g$  = acceleration by gravity ( $980 \text{ cm/sec}^2$ ), and (2) the surface forces holding the oil drop to the pipette ( $= 2r\pi T$ ). Here,  $r$  = radius of the orifice (cm) and  $T$  = oil-water interfacial tension (dynes/cm).

If  $V \Delta d g > 2r\pi T$ , the drop will fall away. Accordingly, for the drop to remain attached, the following relationship must be maintained:  $V \Delta d g < 2r\pi T$ .

Experimental observations have shown that micropipettes with large orifices can maintain larger drops than micropipettes with smaller openings at the microtip. Moreover, oils with higher oil-water interfacial tensions can form larger, stable drops than oils with low values of  $T$ .

With the oil drop immersed in clean water or in inorganic salt solutions, the drop maintains a spherical shape until it is completely retracted following the application of a negative pressure with the drop-retractor (FIGURE 5). A strikingly different situation will develop if the oil first comes in contact with an aqueous phase containing proteins. As the drop is retracted by reducing the back pressure, the sphericity of the drop can be maintained up to a certain degree, the critical diameter (FIGURES 4,B and 6,A). If the drop is made any smaller, the surface becomes crinkled (FIGURES 4,C and 6,B), thereby producing the Devaux effect. The crinkling can be explained by referring to FIGURE 3.

If the retraction is done properly, none of the protein molecules adsorbed at the interface (FIGURE 3,A) can enter the pipette with the oil since the lip of the micropipette serves as a barrier. This arrangement is analogous to the straight-edge barrier used in a conventional Langmuir film trough. The method is essentially a micro-adaptation of the procedure originally developed by Devaux<sup>11</sup> for measuring film characteristics of protein monolayers at liquid-liquid interfaces. In Devaux's method, the area of the interface was expanded or reduced by raising or lowering the water level in the bulbous portion of a flask.

FIGURE 5 (See opposite page.)

A moist chamber, mounted on the stage of a microscope, supports a coverglass from which is suspended a small volume (20 to 40  $\text{mm}^3$ ) of aqueous phase in the form of a hanging drop. The moist chamber, by avoiding evaporation or condensation, maintains the concentration of the components in the aqueous phase at a constant level for considerable periods.

The micropipette and its holder are mounted on the left side of the Chambers' micromanipulator. With this instrument, the micropipette can be moved with precision to any region within the microscopic field.

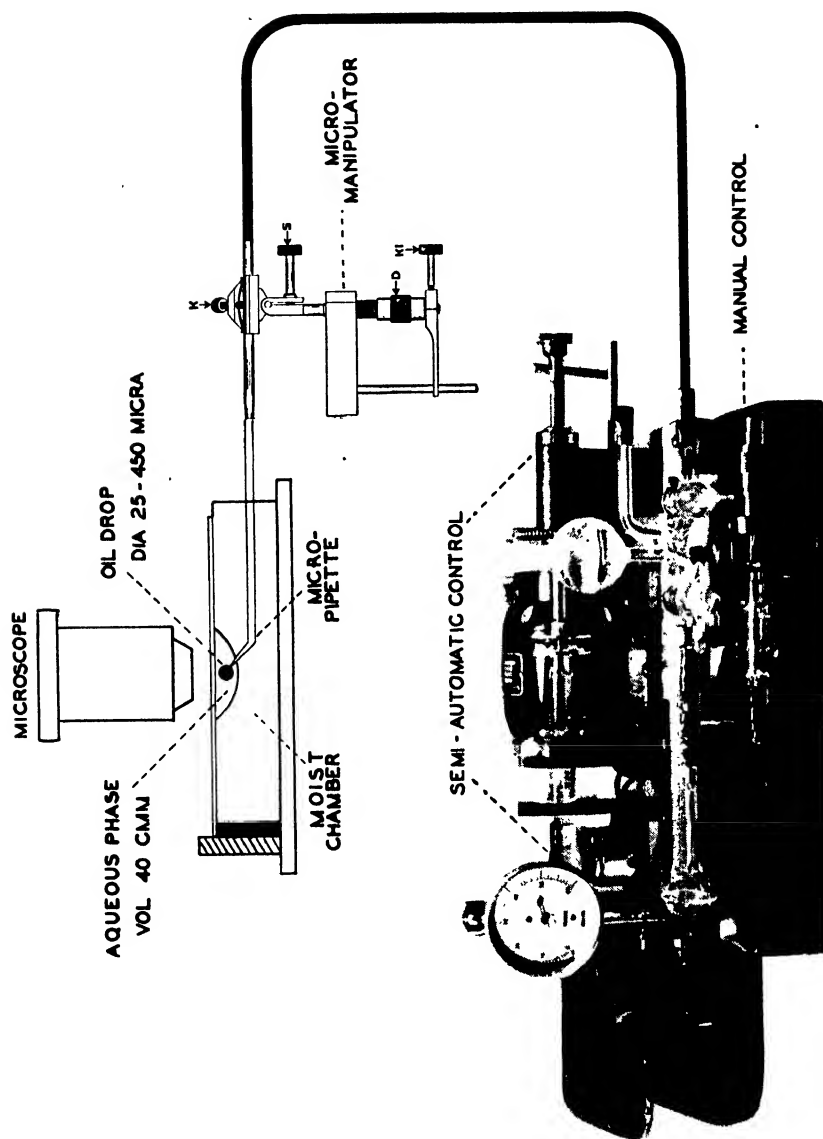
A fine-bore, flexible copper tube joins the micropipette to the drop-retractor. The metal tube, pipette holder, and syringes (on drop-retractor) are filled with distilled water. The volume within the shaftlet and the shaft of the micropipette is filled with air, thereby providing a small compressible volume within the system.

The diameter of an oil drop, poised on the tip of the micropipette, can be precisely controlled by the drop-retractor, an instrument consisting of the following parts: (1) manual control, a 2 ml Luer syringe activated by a micrometer head, (2) semi-automatic control, a 5 ml Luer syringe activated by a slow-speed, motor-driven feed screw, (3) stopcocks and reservoir for filling syringes and connecting tubes with water, and (4) gauge for indicating pressure in the micropipette.

The small gas volume within the shaft of the micropipette can be compressed or expanded by appropriate movements either of the micrometer head or of the motor-driven feed screw. Negative pressure will cause the oil to flow into the pipette and positive pressure will drive it out again. Both the micrometer head and motor-driven feed screw are sufficiently fine so that the diameter of the oil drop can be reduced in steps of less than 1 micron.

The manual control is employed in filling the micropipette, through the tip, with oil from a hanging drop of oil mounted on the moist chamber. This volume will make up the oil drop less the small residuum (FIGURE 6) that must be left in the microtip so that the oil mass may be subsequently retracted.

With the pipette charged with oil and the aqueous phase in place, the tip of the micropipette is inserted into the hanging drop. Pressure is built-up by manipulating the micrometer head to expel the oil from the micropipette. The semi-automatic control is used to retract the oil drop. The motor is operated either with a foot switch or by the electric timer (FIGURE 8).



### DROP-RETRACTOR

FIGURE 5. Pictograph of the drop-retraction apparatus. (For description see facing page.)



An oil drop of diameter,  $D$ , in contact with the aqueous phase presents an interfacial area at which protein molecules may undergo surface denaturation (FIGURE 3,A). This oil drop, at any time, can be retracted to the point that crinkling would occur providing the drop were further reduced in size. On reducing the drop slightly below the critical diameter (FIGURE 6,A), the drop shifts in position indicating a tendency of the drop to fall away from the pipette.

Before retraction,  $V_o \Delta d g < 2r\pi T_o$  ( $V_o$  = volume of drop of diameter,  $D$ , and  $T_o$  = oil-water interfacial tension), but, as  $T$  is diminished by the interfacial concentration of protein molecules, the following situation arises:  $V_i \Delta d g > 2r\pi T_i$  ( $T_i$  = oil-water interfacial tension at time of retraction,

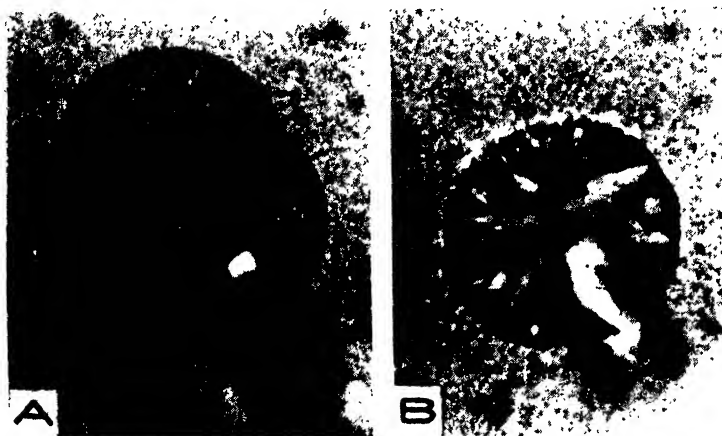


FIGURE 6. Photographs of oil drops in contact with an aqueous phase containing cytoplasmic proteins and granules isolated from sea-urchin eggs.

The intentional double exposure (A) shows the smallest diameter of an oil drop which, under a given experimental condition, remains spherical, and the appearance of the drop when the critical diameter is slightly exceeded. Note that the spherical drop is poised on the end of the micropipette. This represents the critical diameter,  $d$ .

On reducing the drop slightly below the critical diameter, the drop shifts in position. In all instances, the drop quivers at the instant the critical diameter is reached, thus providing an index so that the exact critical diameter may be measured.

Further retraction of the oil mass results in the crinkled surface shown in B. The interfacial film is rigid. While the crinkled state is maintained, it is difficult to separate the oil mass from the micropipette.

These photographs illustrate the principle of the Devaux effect, *e.g.*, the crinkling is produced by a spontaneous expansion of the interface.

and  $V_i$  = volume of oil drop at the same time). This situation is indicated by the appearance of instability and is shown by displacement in position of oil drop (FIGURE 6,A). The diameter of the oil drop at the instant of quivering is taken as the critical diameter. The drop quivers at the instant the critical diameter is reached, thus providing an index so that the exact critical diameter,  $d$ , can be measured.

The critical interfacial area,  $s = \pi d^2$ , represents the total area of protein molecules that cannot be driven out of the oil-water interface by application of surface pressures ranging from 20 to 50 dynes/cm.\* The magnitude of the

\* The drop-retraction method produces a surface compression of the fixed barrier type. The spreading force,  $F$ , is approximately equivalent to the following:  $F = T_o - T_x = nk/(\pi d^2 - nA)$ . Here,  $T_x$  and  $T_o$ , are the interfacial tensions of the oil-water interface, with and without protein molecules;  $k$  = Boltzmann's constant;  $t$  = absolute temperature;  $\pi d^2$  = surface area of oil drop of diameter,  $d$ ; and  $nA$  = area occupied by  $n$  molecules at the interface.

surface forces depends mainly on the components of the oil-water interfacial system. The interfacial area,  $s$ , at any time, includes protein molecules that may be completely unfolded and also portions of molecules that cannot be expelled from the interface, although these molecules may not be entirely unfolded.

The maximum area available for non-displaceable protein molecules is represented by  $A = \pi D^2$ . Accordingly, a convenient means of indicating the extent of surface denaturation of protein molecules is by the fraction,  $s/A$ . This fraction is evaluated by calculating the ratio,  $\pi d^2/\pi D^2$ .

The values of  $s/A$ , at any time, give an estimate of the area of unfolded or surface denatured molecules per unit area of interfacial surface. The value of  $s/A$  may also include portions of the partially unfolded molecules. Succeeding values of  $s/A$  will include those molecules present during preceding measurements plus those that have become unfolded since then. In most instances, molecules that have become completely unfolded represent those that were only partially unfolded during previous measurements. The changes of  $s/A$  with time depend on the oil-water interface, the type of proteins, pH and ionic strength of the aqueous phase, and other factors.

The equations relating interfacial denaturation to time become:

$$s/A = kt^a, \text{ or } \log s/A = \log k + a \log t \text{ (FIGURE 7).}$$

At  $t = 1$ ,  $\log s/A = \log k$ , while  $a =$  the slope, or  $\tan \theta$ , of the  $\log s/A$  versus  $\log t$  curve. All values can be obtained from the logarithmic graph, and one may, therefore, use the values of  $k$  and of the slope,  $a$ , in comparing the surface denaturation of proteins under various interfacial conditions.

Preliminary results (Kopac<sup>5</sup>) from a new series of measurements suggest that a major fraction of the increase in  $s/A$  is due to the progressive unfolding of protein molecules at the interface. Prior to these recent measurements, the increase in  $s/A$  was ascribed to either one of two possibilities: (1) increase in number of protein molecules unfolding at the interface or (2) increase in unfolding of molecules initially present at the interface.

The second possibility now appears to be more probable since the changes in  $s/A$  on oil drops left in media containing bovine plasma albumin molecules were not appreciably different on transferring oil drops to an albumin-free medium. These data suggest that an increase in number of molecules capable of unfolding at the interface is not especially significant since, on transfer to the protein-free medium, the probability of increasing the number of such molecules is drastically reduced. It would appear, therefore, that the increase in  $s/A$  must result from the further unfolding of those molecules acquired by the interface during the early sojourn of the oil drop in an albumin-containing medium.

In some instances,  $k$  may actually represent the initial adsorption and preliminary unfolding of proteins present at the interface, while the slope,  $a$ , is a measure of the rate of unfolding of those molecules initially adsorbed.

The protein molecules present, at certain oil-water interfaces, may unfold enough during the first minute so that they cannot be entirely displaced on drop-retraction. The unfolded portion of these molecules would remain

in the interface on drop-retraction while less unfolded portions might be forced out. This quantity would be expressed by  $k$ .

On re-expansion of the oil drop, the displaced portions would reoccupy interfacial space, and thereby prevent new molecules from entering the interface. With time, more of each molecule would become non-displaceable on drop-retraction. This would be shown by the slope,  $a$ .

Where  $k$  is high, the slope,  $a$ , may not be a true indication of the rate of unfolding, owing to space difficulties involved in subsequent unfolding of the molecules when the interface becomes crowded.

With low  $k$  and high  $a$  values, the situation may be complex. Thus  $k$  is undoubtedly an indication that both the number of molecules and their

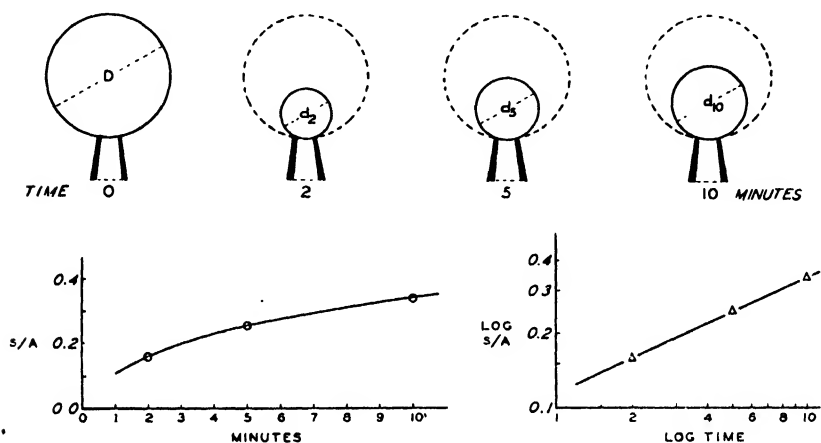


FIGURE 7. Quantitative aspects of the drop-retraction method.

As soon as the oil drop was brought in contact with the aqueous phase, the time was set at zero and its diameter,  $D$ , measured. At 2 minutes, the drop was retracted to its critical diameter,  $d_2$ , and measured. Immediately thereafter, the drop was expanded to its original diameter,  $D$ . At 5 minutes, the drop was again retracted to its new critical diameter,  $d_5$ , measured, and re-expanded to diameter,  $D$ . At 10 minutes, the new critical diameter,  $d_{10}$ , was measured, and so on. If subsequent measurements are to be taken, the drop must be re-expanded to its original diameter,  $D$ . Usually, these measurements are terminated after 15 minutes but some have been continued for periods as long as 90 minutes.

The values of  $s/A$  are calculated for each value of  $d$  as measured at the various times, since  $s/A = \pi d^2 / \pi D^2$ . If one plots the values of  $s/A$  against time, a parabolic curve is obtained as shown in the left graph. A straight line is obtained on plotting  $\log s/A$  against  $\log t$  (right graph).

degree of unfolding is low. The subsequent high value for the slope,  $a$ , may be accounted for in one of 3 ways: (1) an increase in number of molecules that become adsorbed with time, (2) increase in the degree of unfolding of those molecules present at  $t = 1$ , and those acquired later, or (3) a combination of the first two.

The crinkled state is developed on further compression of the interface by reducing drop diameter (FIGURES 4,C and 6,B). It is not difficult to understand that a point can be reached where the interface becomes completely packed with unfolded and trapped protein molecules (FIGURE 3,B). The unfolded molecules are so oriented that the hydrophobic surfaces (non-polar amino acid residues) are directed towards the organic phase, while the hydrophilic surfaces (polar amino acid residues) are directed towards the aqueous phase. Such molecules become trapped at the phase boundary

since they are no longer soluble in the aqueous phase, nor are they soluble in the oil phase. Further reduction of the interfacial area becomes impossible and any attempt to do so results in the crinkling effect (FIGURES 3,C and 6,B).

Since drop-retraction increases the interfacial concentration of surface denatured proteins, the value of  $T$  will be lowered thereby (Kopac<sup>10</sup>). As the critical diameter is approached, the surface concentration of unfolded proteins increases and  $T$  becomes correspondingly reduced and may approach zero. This situation is indicated by the appearance of instability, *i.e.*, quivering (FIGURE 6,A).

Failure of the drop to fall away, at this time, is a result of the elastic nature or yield value of the surface denatured proteins which, when concentrated and compressed, form a membrane of appreciable mechanical strength (FIGURE 6,B).

While the crinkled state is maintained, it is difficult to separate the oil mass from the tip of the micropipette. These data indicate that proteins not only become surface denatured but, on compression, they may also become surface coagulated. The yield values of such protein films are high and compensate remarkably for the low (real) interfacial tension that is developed when the critical diameter is reached or exceeded.

The quantitative aspects of the drop-retraction technique are explained in FIGURE 7. The values of  $s/A$  may cover a range from 0 to 1.0. If  $s/A = 0$ , no surface denaturation has occurred; if  $s/A = 1.0$ , the interfacial area is entirely occupied by non-displaceable, surface denatured protein molecules.

The Devaux effect that develops following cytolysis of a cell at appropriate oil-water interfaces is produced by the interfacial adsorption and denaturation of protein molecules. Under such conditions, the value of  $s/A = 1.0$ , and this value is reached spontaneously. Where protein adsorption and unfolding is less pronounced, the spontaneous Devaux effect does not form, and the degree of surface denaturation may be estimated only by the application of the drop-retraction procedure as shown in FIGURES 5 and 7.

#### *Application of the Drop-Retraction Technique to Cells*

As previously stated, the longer the time interval between cytolysis and application of the oil, the lower is the probability of obtaining spontaneous Devaux effects (FIGURE 2). In fact, after a few minutes have elapsed, only a slight crinkling may develop at the interface (FIGURE 1,D), and, after about 5 minutes, no changes on the surface of the oil may be observed. The question remains whether the substances that ordinarily produce the Devaux effect disappear completely or whether the concentration diminishes with time. To determine satisfactory conclusions on this point, the drop-retraction method was employed, since the technique is sufficiently refined so that it can be used within the dimensions of a single cell.

In FIGURE 9,A the micropipette can be seen with an attached oil drop placed near the margin of an *Asterias* oocyte introduced about 5 minutes after cytolysis. After 10 minutes' exposure to the cytolysed residue, the drop

was retracted until a partial Devaux effect was obtained. In this example, the value of  $s/A$ , as calculated from the two surface areas (FIGURE 7) was *ca.* 0.9. This value indicates that a considerable amount of protein was surface denatured at the oil-water interface, but this amount was insufficient to produce a spontaneous Devaux effect ( $s/A = 1.0$ ).

In general, the longer the period between cytolysis and application of the oil, the smaller will be the value of  $s/A$  as calculated from the measured

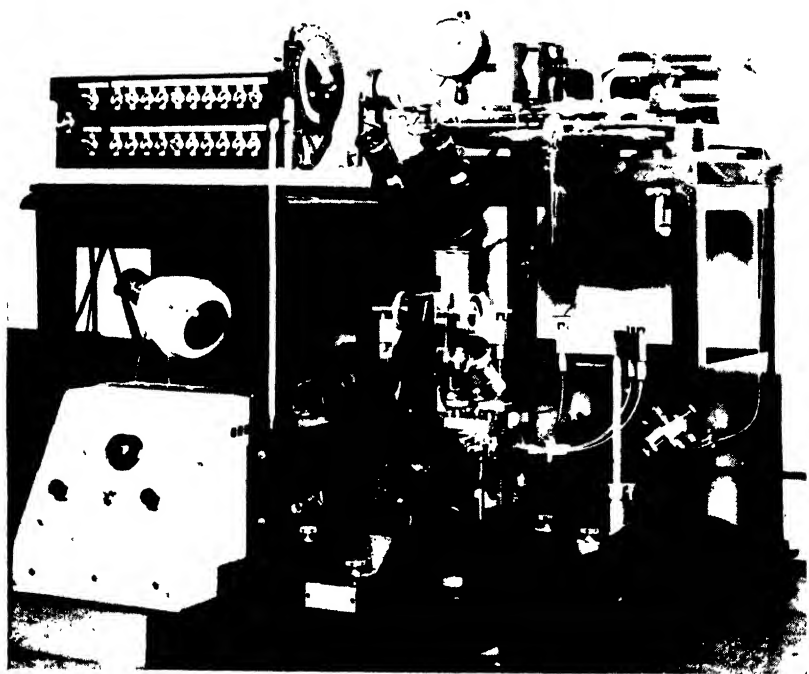


FIGURE 8. The actual apparatus is shown in the above photograph. The positions of the microscope, micromanipulator, and the drop-retractor were arranged to afford maximum convenience for operation.

To maintain time schedules, an electric timer was designed and built (mounted to the left of the micromanipulator and drop-retractor). This unit sets into operation the semi-automatic component (motor-driven syringe, FIGURE 7) of the drop-retractor at pre-determined time schedules, for example, at 2, 5, and 10 minutes after the interface is formed.

It will be noted that the timer as well as the drop-retractor are placed at eye level in order to facilitate observation of these instruments as well as to measure the oil drop visible in the microscopic field.

The optical equipment employed in measuring the oil drops includes: Leitz No. 5 objective, Zeiss 10X oculars, with micrometer disk, and a Zeiss 1.5X Bitukni attachment. These optical units provide a magnification of 450X.

diameters. These data indicate that proteins are still present in the cytolytic residue, but the amounts seem to decrease with time so that fewer molecules are available to the introduced interface. In fact, proteins may be detected by the drop-retraction method in the surrounding sea water when cytolized eggs have been left standing in a hanging drop for several minutes. It is obvious that the proteins have diffused out of the cytolized residue into the surrounding medium.

Such outward diffusion tends to diminish the concentration near the zone of the injected oil. It is reasonable to conclude that the maximum available concentration of protein occurs at the instant of cytolysis. At this time, protein adsorption on suitable oil-water interfaces may be readily demonstrated—by the formation of spontaneous Devaux effects.

Another factor that must be taken into account is the possible role of surface-denaturing agents (see FIGURE 15). Accordingly, the surface denaturation of cytoplasmic proteins would diminish with time providing the surface-denaturing agent ( $x$  in FIGURE 15) either diffused away or became bound by other products of cytolysis. This topic will be discussed in greater detail later on.

Another interesting application of the drop-retraction technique is shown by FIGURE 9,C and D. It is possible to microinject a drop of oil into the cytoplasm of a living *Asterias* oocyte and to maintain continuity between the oil drop and pipette. It will be noted in FIGURE 9,C that the oil drop was placed near the germinal vesicle without inducing the characteristic cytolytic reaction that usually follows any mechanical injury to this structure. After 10 minutes' exposure of the oil-water interface to the cytoplasm, the drop was retracted until a crinkling effect was obtained (FIGURE 9,B). The  $s/A$  value for this experiment was *ca.* 0.1. It will be further noted that the germinal vesicle remained intact and that a small cytolytic zone was present around the retracted oil drop.

Some surface denaturation of proteins occurred at this oil-water interface, even though the cell remained reasonably intact. The small cytolytic zone that surrounded the retracted oil drop probably accounts for all the protein that has become surface denatured. It is obvious that enough protein was not surface denatured to produce a spontaneous Devaux effect.

The changes that occur on cytolysis in the protein structure of the cell will be discussed later (see also FIGURE 15).

#### *Measurements on Model Protein Systems*

The interpretation of the properties of cytoplasmic proteins on the basis of spontaneous Devaux effects must depend on information obtained from a study of individual protein systems. Up to now, over 60 different organic phases have been investigated. The interfacial denaturation at oil-water interfaces of various proteins and nucleoproteins has also been measured.<sup>5, 14, 15, 17</sup> Included in the list of proteins are: bovine and human plasma albumins, thrombin (beta-globulin), crystalline trypsin and chymotrypsin, crystalline ribonuclease, hyaluronidase, protamine, protamine-nucleate complexes, liver nucleoproteins, thymus nucleohistone, tobacco mosaic virus nucleoprotein, and lyophilized cytoplasmic residue from sea-urchin eggs (fraction rich in cytoplasmic ribonucleoproteins).

The action of various chemical agents on interfacial denaturation of these proteins has also been investigated.\* Of particular interest are the compounds that either enhance or inhibit surface denaturation. A few examples will be described, since these results may aid in the interpretation of the surface chemical behavior of proteins, in general, and of cytoplasmic

\* Some of this material was also presented at the AAAS-Gibson Island Research Conference on Cancer, August 12, 1946, an abstract of which appeared in *Cancer Research*. 7: 44. 1947.

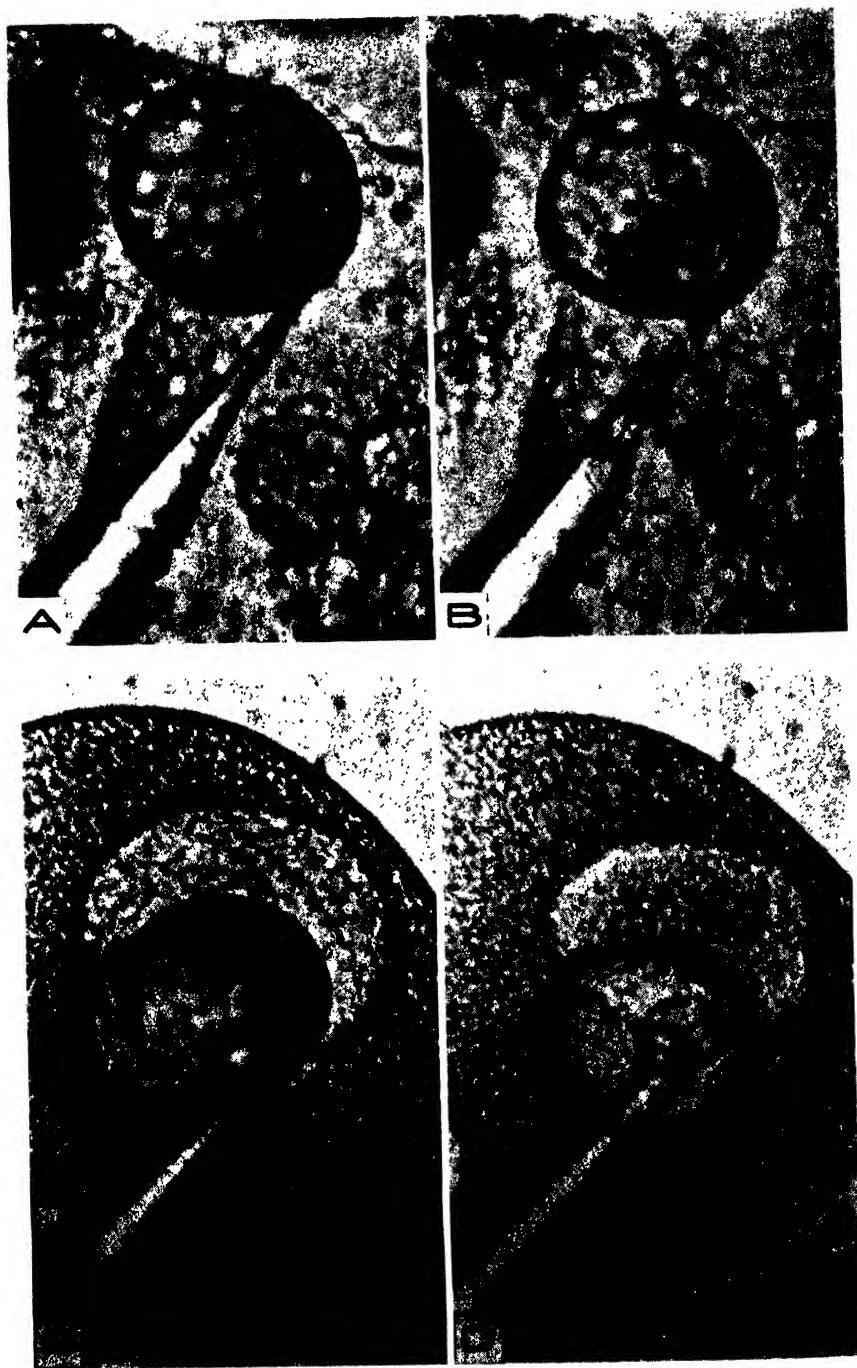


FIGURE 9. (For description see facing page).

proteins, in particular. Certain aspects of protein structure will also be discussed.

*A. The Effects of Surface Forces and of Diamidines on the Surface Denaturation of Liver Nucleoproteins.*

The following data are presented to illustrate the effects of different types of surface forces. The magnitude of the surface forces can be readily controlled by altering the nature of the oil-water interface, *e. g.*: different oil phase, or by changing the pH or ionic strength of the aqueous phase. In the following series, the surface forces were varied by employing different organic liquids to produce the oil-water interfaces.

These data also show the effects produced by a combination of factors, *e. g.*, surface forces + chemical agents. The aromatic diamidines, either stilbamidine\* or propamidine†, at 0.001M, were added to the liver nucleoprotein. The concentration of liver nucleoprotein employed in the measurements summarized in FIGURE 10 was 1.25 mg./ml. The nucleoprotein was dissolved in a saline solution of ionic strength = 0.02, buffered at pH 7.7.

*Tricaprylin + Hexadecanoic Acid‡-Water Interface.* For liver nucleoprotein alone, the value of  $k = 0.062$  and the slope,  $a = 0.54$ . In the presence of stilbamidine,  $k$  increased to 1.0. On the other hand, propamidine reduced the value of  $k$  to 0.0038. Indeed, the values of  $s/A$  were not measurable during the first 3 or 4 minutes. The lowest value of  $s/A$  that can be measured with the present experimental equipment is about 0.02.

*Iso-amylaniline-Water Interface.* The qualitative effects of the two diamidines on the surface denaturation of liver nucleoprotein resembled those obtained with tricapyrin + hexadecanoic acid. There were significant quantitative differences, however. There was more surface denaturation of liver nucleoprotein at this interface as shown by a  $k$  value of 0.16 ( $a = 0.33$ ). Furthermore, the enhancing effect of stilbamidine or the depressing effect of propamidine on the surface denaturation of liver nucleoprotein was much less striking.

*Ethyl Formyl Ethyl Xanthate-Water Interface.* Considerable amounts of liver nucleoprotein were surface denatured at this interface,  $k = 0.8$  and  $a = 0.083$ . Stilbamidine produced a slight increase while propamidine mildly depressed the surface denaturation. The surface forces at this interface are too high for liver nucleoprotein, consequently not much can be learned from the simultaneous action of surface forces and diamidine with this interfacial system.

\* 4,4'-diamidinostilbene diisethionate.

† 4,4'-diamidinodiphenoxypropane diisethionate.

‡ The mol fraction of hexadecanoic acid in tricapyrin was 0.01.

FIGURE 9 (See opposite page). Application of drop-retraction technique to cells.

A. A micropipette with attached oil drop was placed into the cytolized residue near the margin of an *Asterias* oocyte, approximately 5 minutes after cytolysis. Note outpouring of granules through the torn membrane of oocyte.

B. After 10 minutes' exposure to the residue, the drop was retracted until an incipient crinkling was developed at the oil-water interface. In this experiment, the value of  $s/A$ , as calculated from the two surface areas (see FIGURE 7), is ca. 0.9.

C. An oil drop was carefully injected into the cytoplasm of an intact *Asterias* oocyte, near the region of the germinal vesicle (note the prominent nucleolus). The micropipette was not removed, so that continuity between the oil drop and the pipette could be maintained.

D. After 10 minutes' exposure, the oil drop was retracted until the crinkling stage was developed. The value of  $s/A$  in this experiment is ca. 0.1. Note the small cytolytic zone around a portion of the retracted oil drop produced by the original injection of the oil drop shown in C. The residue resulting from the small cytolytic zone was probably the source of the proteins surface denatured at this oil-water interface.



*Ethyl Oleate-Water Interface.* Although the surface denaturation of liver nucleoprotein at this interface was nearly identical to that observed with *iso-amylaniline-water* interfaces, the action of the diamidines was different. Both stilbamidine and propamidine depressed the surface denaturation of liver nucleoprotein at this interface. The depressing action of propamidine, however, was considerably greater than that produced by stilbamidine.

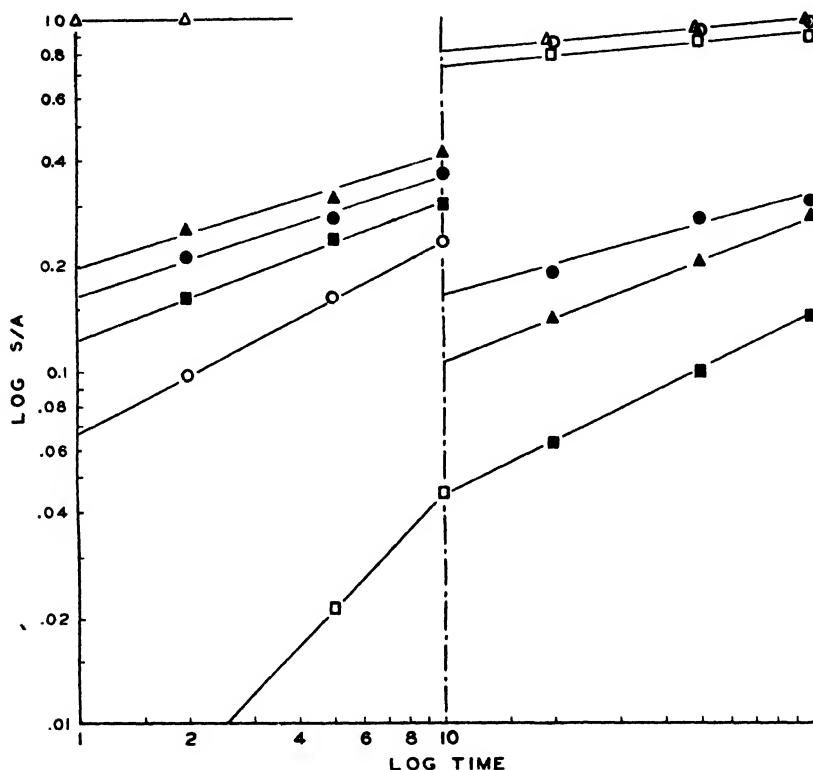


FIGURE 10. The effects of surface forces and aromatic diamidines on the surface denaturation of liver nucleoprotein. *Log s/A vs. log time, in minutes.*

*Left graph. Tricaprylin (hexadecanoic acid)-water interface. Open ovals: LN\*. Open triangles: LN + stilbamidine (0.001M). Open squares: LN + propamidine (0.001M). Iso-amylaniline-water interface. Solid ovals: LN. Solid triangles: LN + stilbamidine (0.001M). Solid squares: LN + propamidine (0.001M). Right graph. Ethyl formyl ethyl xanthate-water interface. Open ovals: LN. Open triangles: LN + stilbamidine (0.001M). Open squares: LN + propamidine (0.001M). Ethyl oleate-water interface. Solid ovals: LN. Solid triangles: LN + stilbamidine (0.001M). Solid squares: LN + propamidine (0.001M).*

\* Liver nucleoprotein (LN) in all preparations: 1.25 mg./ml in NaCl-solution of ionic strength = 0.02, pH = 7.7.

These data indicate that surface forces as well as chemical agents are of importance in the surface denaturation of liver nucleoprotein. The most striking differences between the action of stilbamidine and of propamidine were produced at the *Iricaprylin (hexadecanoic acid)-water* interface.

*B. Action of Aromatic Diamidines and Other Compounds in Modifying the Stilbamidine Effect on Liver Nucleoprotein at the Tricaprylin (Hexadecanoic Acid)-Water Interface.*

The data, summarized in FIGURE 11, were obtained with liver nucleoprotein (1.25 mg/ml.) dissolved in NaCl-solution of ionic strength = 0.02, buffered at pH 7.7 (Kopac<sup>14</sup>).

With stilbamidine, at concentrations as low as 0.0005M, the surface denaturation of liver nucleoprotein at the *tricaprylin* (*hexadecanoic acid*)-*water* interface increased from  $k = 0.092$  to 0.215 and from  $a = 0.34$  to 0.90. The following data show the action of other compounds on this

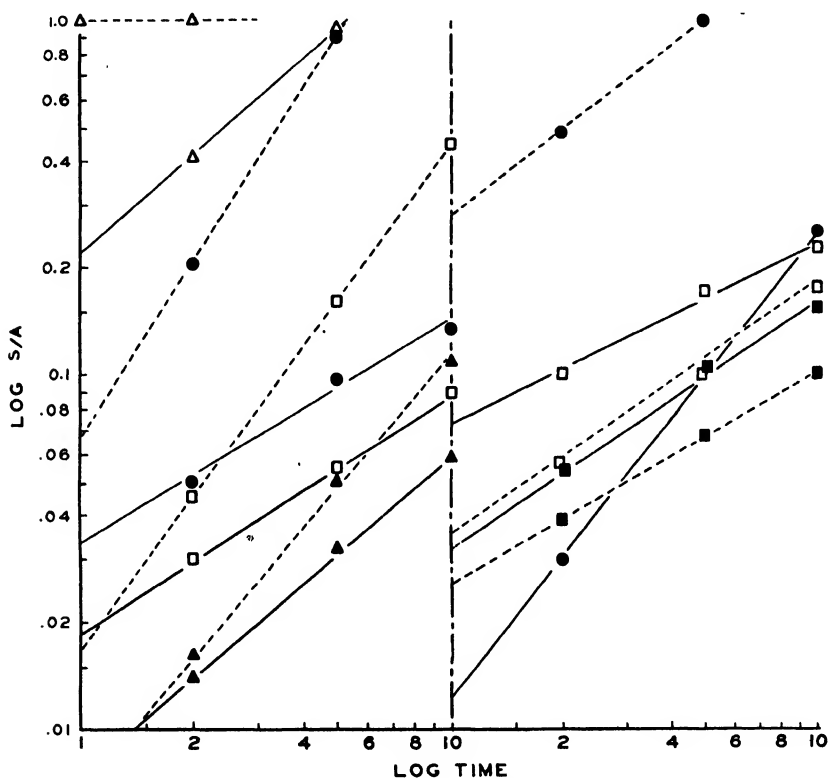


FIGURE 11. Action of aromatic diamidines and other compounds in modifying the stilbamidine effect on liver nucleoprotein at *Tricaprylin* (*hexadecanoic acid*)-*water* interface.  $\log s/A$  vs.  $\log$  time, in minutes. *Left graph.* Open triangles: LN + stilbamidine (0.0005M). Open squares: LN + propamidine (0.0005M). Solid ovals: LN + phenamidine (0.0005M). Solid triangles: LN + pentamidine (0.0005M). *Right graph.* Solid ovals: LN + colchicine (0.001M). Open squares: LN + 1,2-diphenylethylamine (0.001M). Solid squares: LN + 1,2-di-*p*-anisylethylamine (0.001M). Broken lines, ---, indicate changes in  $\log s/A$  following the addition of stilbamidine, at 0.0005M to each preparation.

\* Liver nucleoprotein (LN) in all preparations: same as in FIGURE 10.

effect. The measurements were obtained by adding stilbamidine, at 0.0005M, to liver nucleoprotein previously treated with one of the other compounds.

**Aromatic Diamidines.** The aromatic diamidines were employed at concentrations of 0.0005M. On adding 0.0005M of stilbamidine to a preparation already containing this amount (total concentration = 0.001M), the surface denaturation increased to  $k = 1.0$ .

Phenamidine\*, propamidine, and pentamidine†, depressed the stilbamidine effect in proportion to their individual depressing actions on the surface denaturation of liver nucleoprotein. The  $k$  values were the following: pentamidine = 0.0068, propamidine = 0.165, and phenamidine = 0.64.

With propamidine or pentamidine, the  $k$  values were lower with stilbamidine added, but the slopes were much higher. The slope,  $a$ , for propamidine increased from 0.67 to 1.41 and for pentamidine from 0.88 to 1.48. The  $k$  value for phenamidine was increased on adding stilbamidine, from 0.31 to 0.64, and the slope,  $a$ , was likewise increased, from 0.62 to 1.63.

In the data summarized in FIGURE 11, stilbamidine was added after the other diamidines were allowed to act on the liver nucleoprotein. The question came up whether or not any of these compounds could block the stilbamidine effect if they were added after the nucleoprotein had been exposed to stilbamidine. One such series was measured with propamidine and stilbamidine. In one set, propamidine was added following stilbamidine, and in another, stilbamidine was added following propamidine. The  $k$  values for the two systems were nearly identical. The slope,  $a$ , of the curve for stilbamidine + propamidine was slightly lower than for the curve of propamidine + stilbamidine (1.26 and 1.44, respectively).

*Colchicine.* Colchicine, at 0.001M, + stilbamidine promoted a higher degree of surface denaturation than stilbamidine alone. Stilbamidine increased the  $k$ -value of colchicine from 0.12 to 0.29 (compared with 0.21 for stilbamidine alone), but the slope,  $a$ , was reduced from 1.33 to 0.93. Colchicine was the only compound of the many tested that augmented the surface denaturing action of stilbamidine on liver nucleoprotein.

It will be noted (FIGURE 11) that colchicine alone, at 0.001M, produced a  $k$ -value of 0.013 and a slope,  $a$ , of 1.33. Thus, protein unfolding was inhibited at first, but this effect was rapidly dissipated as shown by the high value of the slope,  $a$ . In general, the value of  $k$  represents initial adsorption and preliminary unfolding of the proteins that are exposed to interfacial forces for one minute. The value of the slope,  $a$ , is generally assumed to be a measure of the rate of unfolding of the initially adsorbed protein molecules at the oil-water interfaces (Kopac<sup>5</sup>).

The high slopes produced by colchicine, however, could be realized only if additional molecules unfolded at the interface, since the low value of  $k$  would not account for the increased interfacial area,  $s$ , occupied by the surface denatured proteins, for example; at time = 10 minutes. This represents a 19-fold increase in the area,  $s$ , and  $k$  could account for only about 20 per cent of this increase.<sup>5</sup>

The curves of depressor diamidines + stilbamidine have slopes similar to those measured with colchicine. These data suggest that some linkages can be weakened, *i.e.*, by stilbamidine, while others can be strengthened, *i.e.*, by propamidine, pentamidine, or by phenamidine (see also discussion of tobacco mosaic virus nucleoprotein). When two diamidines of different properties are present simultaneously, the combined effect will be a resultant

\* 4,4'-diamidinodiphenylether dihydrochloride.

† 4,4'-diamidinodiphenoxypentane dihydrochloride.

between the weakening characteristics of stilbamidine and the strengthening characteristics of the other diamidines.

Thus, the combined action of propamidine + stilbamidine, for example, quantitatively duplicates the effects produced by colchicine. Colchicine, therefore, appears to have double action, *i.e.*, simultaneous weakening and strengthening action of side chain linkages, with the former effect predominating when stilbamidine is present.

On the other hand, colchicine may inhibit initial adsorption (responsible for low  $k$ -values) and unfolding of liver nucleoprotein molecules, but it does not prevent accumulative adsorption and subsequent unfolding (responsible for high  $\alpha$ -values). Possibly, colchicine has an interfacial effect that might prevent extensive adsorption, while at the same time it weakens the protein molecules so that they readily unfold when they reach the interface and are exposed to interfacial forces.

*Phenethylamines.* These compounds form a class by themselves. Either 1,2-diphenylethylamine or 1,2-di-p-anisylethylamine, at 0.001M, protected the liver nucleoprotein molecules against stilbamidine to such an extent that the surface denaturation of liver nucleoprotein was even lower in the presence of stilbamidine than in its absence. Both compounds yielded significantly lower  $k$  values on the addition of stilbamidine. 1,2-diphenylethylamine produced a higher slope on addition of stilbamidine, increasing it from 0.51 to 0.74. 1,2-di-p-anisylethylamine, however, yielded a lower slope, decreasing it from 0.70 to 0.60.

1,2-diphenylethylamine, at 0.001M, did not show any appreciable action on the surface denaturation of liver nucleoprotein. On the other hand, 1,2-di-p-anisylethylamine, at the same concentration, depressed interfacial denaturation to the same degree as phenamidine, at 0.0005M (FIGURE 11).

In the absence of other tests, it could be concluded that either 1,2-di-p-anisylethylamine or phenamidine have a similar action on the liver nucleoprotein molecules. However, on the addition of stilbamidine, at 0.0005M, to the preparations containing either compound, the differences in surface denaturation were striking. Phenamidine + stilbamidine produced a higher surface denaturation than phenamidine alone. On the other hand, 1,2-di-p-anisylethylamine + stilbamidine depressed surface denaturation to a level lower than that caused by 1,2-di-p-anisylethylamine alone. Obviously, phenamidine and 1,2-di-p-anisylethylamine do not have the same action on the nucleoprotein molecules.

The action of these two phenylethylamines on liver nucleoprotein distinguishes them from all other compounds tested. The situation is unusual, in that the addition of stilbamidine actually increases the depressor action of another compound.

More data, particularly on the action of the phenylethylamines on albumin and other proteins will be needed before the curious combined actions of 1,2-diphenylethylamine + stilbamidine or of 1,2-di-p-anisylethylamine + stilbamidine can be explained. The nucleate fraction of the nucleoprotein may be partly responsible. Some suggestions about this effect will be discussed later (section on bovine plasma albumin). Furthermore,

several new aromatic amidines, in combination with nucleic acid, produced new and unpredictable effects on the surface denaturation of bovine plasma albumin (Kopac<sup>15</sup>).

*C. The Action of Aromatic Diamidines and Other Compounds on the Surface Denaturation of Cytoplasmic Proteins.*

The material employed in this series consisted of the proteins of the cytoplasmic matrix, mitochondria, and the smaller yolk granules that may be obtained by rapid disintegration of unfertilized sea-urchin (*Arbacia*) eggs in Ca-free media and by subsequent centrifugal fractionation of such products (Kopac<sup>10</sup>). The mitochondrial content was 5 to 10 times higher than that normally present in unfertilized *Arbacia* eggs. The cytoplasmic

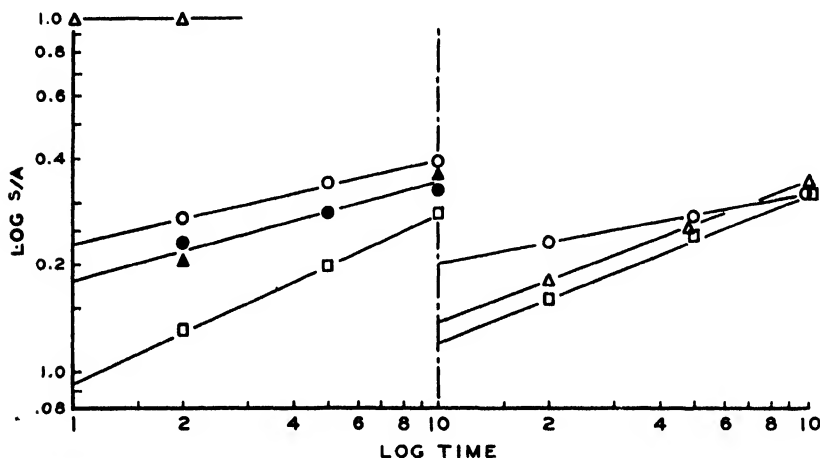


FIGURE 12. The action of aromatic diamidines on the surface denaturation of cytoplasmic proteins. *Log S/A vs. log time, in minutes.*

*Left graph. Tricaprylin (hexadecanoic acid)-water interface.*

*Open ovals:* CP\*. *Open triangles:* CP + stilbamidine (0.001M). *Open squares:* CP + propamidine (0.001M). *Solid ovals:* CP + phenamidine (0.001M). *Solid triangles:* CP + bis-amidinomethylidibenzyl (0.001M).

*Right graph. Tricaprylin-water interface.*

*Open ovals:* CP. *Open triangles:* CP + stilbamidine (0.001M). *Open squares:* CP + propamidine (0.001M).

\* Cytoplasmic proteins (CP): 2 mg./ml., lyophilized weight, in NaCl-solution of ionic strength = 0.15, pH = 7.5.

matrix residue consisted to a large extent of nucleoprotein complexes (see Harvey and Lavin<sup>8</sup>). Following centrifugal fractionation, the material was lyophilized.

The lyophilized cytoplasmic residue, at 2 mg./ml., was reconstituted by dissolving and suspending in a buffered, saline solution of ionic strength = 0.15. The saline solution was maintained at pH 7.5. The data are summarized in FIGURE 12.

Stilbamidine enhanced surface denaturation at the *tricaprylin* (hexadecanoic acid)-water interface, while the other diamidines depressed surface denaturation. Propamidine depressed considerably more than either phenamidine or bis-amidinomethylidibenzyl\*. It is of interest to note that both

\* 4,4'-bis-amidinomethylidibenzyl dihydrochloride.

phenamidine and bis-amidinomethyldibenzyl produced similar effects on tobacco mosaic virus nucleoprotein as well as on cytoplasmic proteins.

At the *tricaprylin-water* interface, the surface denaturing action of stilbamidine was considerably reduced (see also tobacco mosaic virus nucleoprotein). Both stilbamidine and propamidine temporarily depressed surface denaturation. Although their  $k$ -values (0.14 and 0.125, respectively) were lower than that obtained for cytoplasmic residue alone ( $k = 0.20$ ), the slopes,  $a$ , were 0.38 and 0.40, respectively, compared to 0.20 for the cytoplasmic residue alone.

There is considerable evidence to indicate that some of the cytoplasmic proteins, including the mitochondria, are high molecular weight complexes (Claude<sup>19</sup>) and that dissociation of these into smaller units is a prerequisite to subsequent surface denaturation. In several ways, the action of the diamidines on this material was similar to their action on tobacco mosaic virus nucleoprotein (see following section). Cytoplasmic proteins are highly complex and, at present, the simplest models appear to be the virus nucleoproteins.

*D. The Action of Aromatic Diamidines on the Surface Denaturation of Tobacco Mosaic Virus Nucleoprotein (TMV) at Oil-Water Interfaces.*

These data, summarized in FIGURE 13, show the action of several diamidines on a complex nucleoprotein in which the the molecular complex must be dissociated into smaller molecules before surface denaturation can occur (see Seastone<sup>16</sup>). The surface denaturation of complexes of this category, therefore, involves: (1) dissociation of the large molecules or complexes into smaller molecules and (2) unfolding of the smaller molecules by the action of surface forces.

Surface forces of appropriate magnitude may be sufficiently strong to induce both changes and if an agent enhanced the dissociation of the large complex, surface denaturation should be correspondingly increased. If such agents also facilitate unfolding, surface denaturation would be enhanced to an even higher degree.<sup>14, 17</sup>

The results differ from those previously described for liver nucleoprotein. The surface denaturation of TMV (1 mg./ml. in NaCl-solution, ionic strength = 0.02, pH = 7.7) was strikingly enhanced by either stilbamidine, propamidine, or pentamidine, each at 0.001M. The  $k$  values ranged from 0.96 to 1.0.

Phenamidine (0.001M) or bis-amidinomethyldibenzyl (0.001M), on the other hand, depressed the surface denaturation of TMV at the *tricaprylin* (*hexadecanoic acid*)-water interface ( $k$ -values, 0.034 and 0.017, respectively).

The action of surface forces on the denaturation of TMV has also been demonstrated. The surface denaturation of TMV + stilbamidine was tested with several oil-water interfaces. The  $k$  values were highest at the *tricaprylin* (*hexadecanoic acid*)-water interface, and much lower at *tricaprylin-water* or at *tricaprylin* (*stilbestrol*)-water interfaces (0.21, 0.014, and 0.011, respectively). These data indicate that stilbamidine does not enhance unfolding of the dissociation products as much at some interfaces as it does at others. A similar interfacial effect was observed with liver nucleoprotein + stilbamidine at the *ethyl oleate-water* interface (FIGURE 10).

Although propamidine or pentamidine generally depressed the surface denaturation of proteins at oil-water interfaces, their action on TMV may be interpreted in the following way: For example, propamidine, at 0.001M, enhanced the surface denaturation of bovine plasma albumin (BPA) providing the concentration of the latter was not higher than 2 mg./ml.<sup>15</sup> If the concentration of BPA was increased to 5 mg./ml., then propamidine

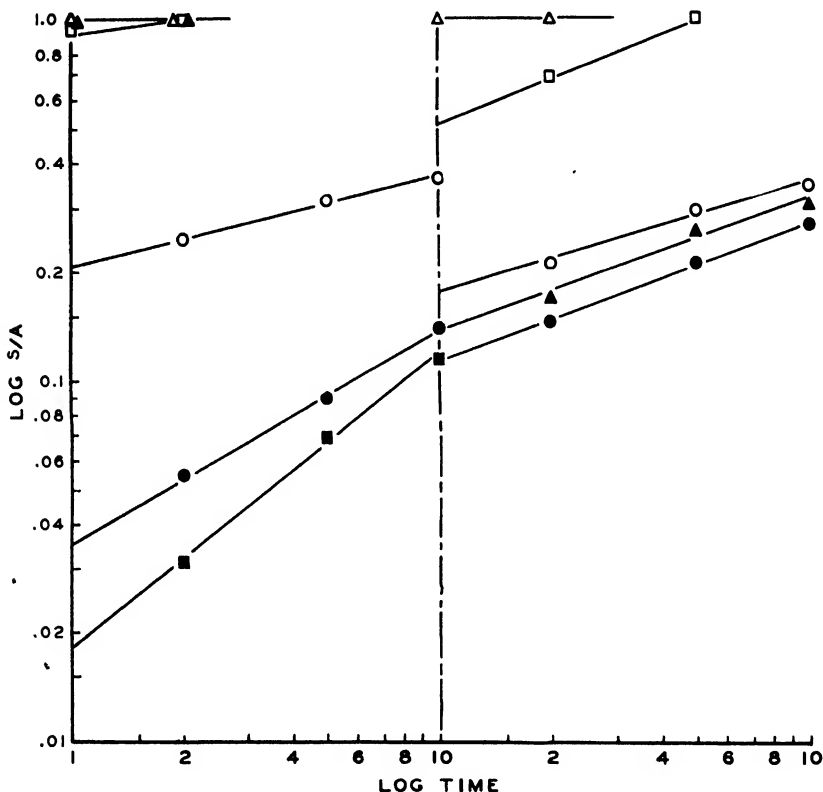


FIGURE 13. The action of aromatic diamidines on the surface denaturation of tobacco mosaic virus nucleoprotein at oil-water interfaces.  $\log s/A$  vs.  $\log$  time, in minutes.

Left graph. *Tricaprylin* (hexadecanoic acid)-water interfaces. Open ovals: TMV\* (1.0 mg./ml.). Open triangles: TMV + stilbamidine (0.001M and 0.0005M). Open squares: TMV + propamidine (0.001M). Solid ovals: TMV + phenamidine (0.001M). Solid triangles: TMV + pentamidine (0.001M). Solid squares: TMV + bis-amidinomethylidibenzyl (0.001M).

Right graph. *Tricaprylin* (hexadecanoic acid)-water interface. Open ovals: TMV (1.96 mg./ml.). Open triangles: TMV + stilbamidine (0.001M). Open squares: TMV + propamidine (0.001M). *Tricaprylin*-water interface. Solid triangles: TMV + stilbamidine (0.001M). *Tricaprylin* (stilbestrol)-water interface. Solid squares: TMV + stilbamidine (0.001M).

\* Tobacco mosaic virus nucleoprotein (TMV): in NaCl-solution of ionic strength = 0.02, pH = 7.7.

depressed surface denaturation at *tricaprylin* (hexadecanoic acid)-water interfaces.

All three aromatic diamidines in this group may be expected to dissociate an appreciable fraction of the TMV complex ( $P^n$ ) to smaller molecules ( $np$ ), the latter being more susceptible to surface denaturing forces. If the concentration of  $np$  molecules is low, then propamidine or pentamidine

may enhance surface denaturation as observed with low concentrations of BPA.

It may, at first, be difficult to understand why one diamidine always enhances surface denaturation while others do so only if the *diamidine/np* ( $= D/np$ ) ratio is high. It has been suggested (Kopac<sup>17</sup>) that stilbamidine enhances surface denaturation because it can weaken or destroy certain critical side chain linkages in the protein molecule.

Crammer and Neuberger<sup>18</sup> postulated that in the native ovalbumin molecule the phenolic groups are bound in linkage, possibly a hydrogen bond. It was suggested that the phenolic group may interact with a carboxyl group, via H-bridges, similar to interaction of phenols with salicylic acid. An amidine group may be expected to rupture a glutamyl-tyrosyl side chain linkage by interacting with the H-bridges. If this should happen, the trans-stilbene structure of stilbamidine could present only one amidine group/stilbamidine molecule for interaction with either the carboxyl or the phenolic groups of the severed side chain linkage. Indeed, if the concentration of stilbamidine were high enough, both end groups of the severed linkage could interact with a pair of amidine groups, but these would have to come from two stilbamidine molecules, and not one. It must be remembered that only one amidine group/molecule can be involved in a severed linkage, since the steric architecture of trans-stilbamidine does not readily permit other possibilities.

Propamidine or pentamidine, on the other hand, could interfere with the same type of side chain linkages, but now it is sterically possible for both amidine groups of one molecule to combine with the phenolic and carboxyl end groups of the severed side chain linkage. Under such conditions, the side chain linkage would not be weakened, rather the phenolic and carboxyl groups would be joined together by the propamidine or pentamidine molecules. Thus, the structure of the protein molecule is not weakened, and it is even possible that a glutamyl-propamidine-tyrosyl linkage might be stronger than the original glutamyl-tyrosyl linkage.

This situation might be expected if the *D/np* ratio were low. With a high *D/np* ratio, enough diamidine molecules are available so that the severed free groups of a glutamyl-tyrosyl linkage, for example, could be joined to two diamidine molecules instead of one. There would be no joining together of the carboxyl and phenolic groups to give a glutamyl-propamidine-tyrosyl linkage, but rather glutamyl-propamidine and tyrosyl-propamidine linkages could be formed. Since no stable junction between the free ends of the two diamidine molecules is to be expected, such a condition would weaken the protein molecule in the same way as proposed for stilbamidine.

That the *diamidine/TMV* ratio is important can be shown by the following data: Where the propamidine concentration was 0.001M and the TMV concentration 1 mg./ml., the value of *k* was 0.96. On doubling the concentration of TMV, for the same concentration of propamidine, the value of *k* fell to 0.50. On the other hand, the surface denaturing action of stilbamidine, at 0.005M, was as high on a TMV concentration of 2 mg./ml., as it was at 0.001M for one-half the concentration of TMV (*k* = 1.0, for both).



According to the general scheme outlined above, there is little, if any, possibility that stilbamidine can reinforce any linkage that it might break. For example, it is sterically impossible to produce a glutamyl-stilbamidine-tyrosyl linkage in the way that a glutamyl-propamidine-tyrosyl linkage can be formed. At all concentrations, stilbamidine weakens the architecture of globular proteins.

Both phenamidine or bis-amidinomethyl-dibenzyl structures could form linkages as described for propamidine and pentamidine. However, judging from the action of these molecules on the surface denaturation of TMV, it would appear that the TMV complexes are strengthened so that surface forces are unable to dissociate appreciable fractions of the giant molecules. Accordingly, surface denaturation of TMV is depressed by these two compounds.

Also, owing to the structure of these two molecules, it would appear that they could readily form glutamyl-phenamidine-tyrosyl or glutamyl-amidino-methyl-dibenzyl-tyrosyl linkages, and that it would be difficult for two diamidine molecules to react with the two liberated terminal groups of the side chains even if the diamidine concentration were high. This seems to be especially true for bis-amidinomethyl-dibenzyl.<sup>5</sup> Thus, these two substances not only protect the large TMV complexes from dissociating, but they also protect the dissociation products from surface denaturing forces. Either condition would account for the depressing action of these compounds on the surface denaturation of TMV.

Bis-amidinomethyl-dibenzyl, at 0.001M, completely inhibited the surface denaturation of BPA, at 2 mg./ml., and of crystalline ribonuclease, at 1 mg./ml. With higher concentrations of BPA (5 mg./ml.), bis-amidinomethyl-dibenzyl strongly depressed surface denaturation, but it did not prevent it, as was observed with lower concentrations of BPA. Owing to the structure of this compound, it would appear that such molecules always react with two side chains to form a new stable side chain linkage e.g., glutamyl-amidinomethyl-dibenzyl-tyrosyl linkage. Accordingly, BPA molecules with glutamyl-amidinomethyl-dibenzyl-tyrosyl linkages are more resistant to surface denaturation than BPA molecules with glutamyl-tyrosyl linkages.

If 0.0005M of stilbamidine was added to TMV treated with either phenamidine or bis-amidinomethyl-dibenzyl, the resulting surface denaturation was the same as with stilbamidine alone ( $k = 1.0$ ). This is similar to the combined action of stilbamidine and bis-amidinomethyl-dibenzyl on BPA in which the stilbamidine effect predominated. Indeed, bis-amidinomethyl-dibenzyl produced no measurable inhibition of the stilbamidine effect on BPA. Obviously, stilbamidine can replace either phenamidine or bis-amidinomethyl-dibenzyl in the TMV complex, either before or after dissociation.

*E. The Action of Aromatic Diamidines on the Surface Denaturation of Bovine Plasma Albumin (BPA) and the Modification of These Effects by Nucleic Acids.*

The following data, summarized in FIGURE 14, were based on measurements of diamidine on the surface denaturation of bovine plasma albumin (5 mg./ml., dissolved in NaCl solution of ionic strength = 0.02, buffered at

pH 7.7). All measurements were made with the *tricaprylin* (hexadecanoic acid)-water interface. These measurements are of particular interest since they illustrate clearly that the action of stilbamidine in enhancing, and other substances in depressing, surface denaturation results from the simultaneous action of surface forces and of chemical agents on the protein molecules.

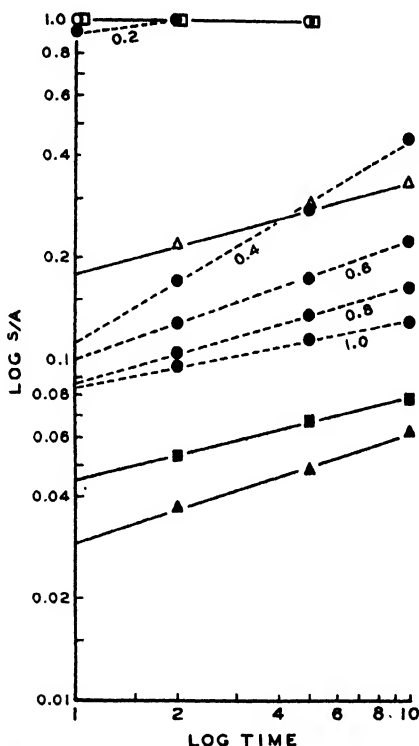


FIGURE 14. Action of stilbamidine, protamine, and nucleic acids on the surface denaturation of bovine plasma albumin at the *Tricaprylin* (hexadecanoic acid)-water interface.  $\log S/A$  vs.  $\log$  time, in minutes.

*Open ovals:* BPA\* + stilbamidine (0.001M). *Solid ovals:* BPA + stilbamidine (0.001M) + Na ribonucleate (0.2 to 1.0 mg./ml.). *Solid triangles:* BPA + protamine (1 mg./ml.) + Na ribonucleate (1.0 mg./ml.). *Open squares:* BPA + protamine (1 mg./ml.) + stilbamidine (0.001M). *Solid squares:* BPA + protamine (1 mg./ml.) + stilbamidine (0.001M) + Na ribonucleate (1 mg./ml.). *Open triangles:* BPA + protamine-ribonucleate (2 mg./ml.) + stilbamidine (0.001M).

\* Bovine plasma albumin (BPA): 5 mg./ml, in NaCl-solution of ionic strength = 0.02, pH 7.7.

It will be noted that stilbamidine, at 0.001M, enhanced the surface denaturation of BPA as it did for liver nucleoprotein, TMV, crystalline ribonuclease, and the cytoplasmic proteins. On adding Na ribonucleate, the action of stilbamidine was neutralized, owing to the formation of a stilbamidine-ribonucleate complex. Na ribonucleate, at 0.2 mg./ml., had a very slight effect in reducing the surface denaturing action of stilbamidine; however, at 0.8 to 1.0 mg./ml., the action of stilbamidine was almost entirely neutralized<sup>16</sup>. The action of stilbamidine on BPA could be partly neutralized also by yeast adenylic acid (1 mg./ml.).

On the other hand, protamine, at 1.0 mg./ml., inhibited the surface denaturation of BPA. The surface denaturation of BPA, on the addition of Na ribonucleate, at 1.0 mg./ml., was considerably higher indicating that an appreciable fraction of the protamine was bound by the formation of a protamine-ribonucleate complex and thereby neutralized.

A typical stilbamidine effect was produced on adding stilbamidine, at 0.001M, to a preparation containing BPA (5 mg./ml.) + protamine (1 mg./ml.). On the subsequent addition of Na ribonucleate, at 1 mg./ml., a typical protamine effect was elicited indicating that stilbamidine was preferentially bound by the ribonucleate.

Stilbamidine, when added to preparations containing BPA (5 mg./ml.) + colloiddally dispersed, protamine-ribonucleate (2 mg./ml.) produced an important effect. The action of stilbamidine was much lower than measured previously for stilbamidine + protamine. These data suggest that the protamine-ribonucleate complex was dissociated by stilbamidine to form a new stilbamidine-ribonucleate complex (Kopac<sup>17, 20, 21</sup>). Thus, the stilbamidine was removed from the reactants and protamine was released from the initial protamine-ribonucleate complex. Accordingly, the surface denaturation of BPA was much less than would be expected if stilbamidine, at 0.001M, alone were acting on the protein molecules.

Previous measurements have shown that protamine can also become trapped at the *tricaprylin* (hexadecanoic acid)-water interface providing it has been treated with stilbamidine.<sup>17</sup> It will be noted that the surface denaturation curve of BPA + protamine-ribonucleate + stilbamidine, in FIGURE 14, is approximately a sum of the curves for BPA + stilbamidine + ribonucleate (0.6 mg./ml.), for BPA + protamine + stilbamidine + ribonucleate, and for BPA + protamine + ribonucleate. It is apparent that the interface contained both surface denatured BPA and protamine.

A stilbamidine concentration of 0.0002M would be required to produce the same degree of surface denaturation with BPA alone. On this basis, about 80 per cent of the stilbamidine was removed by cleavage of the protamine-ribonucleate and subsequent formation of stilbamidine-ribonucleate<sup>6</sup>.

These measurements are helpful in interpreting the spontaneous Devaux effect that occurs in *Asterias* oocytes after cytolysis. The first major point is that the surface denaturation of a protein can be significantly modified by the presence of certain chemical agents. On one hand, surface denaturation can be enhanced (stilbamidine), while on the other hand, the surface denaturation can be almost abolished (protamine). Stilbamidine has the action of a surface denaturing factor, while protamine behaves as an anti-surface denaturing factor.

Another major point is that the simultaneous presence of surface denaturing and anti-surface denaturing factors may produce an effect on surface denaturation that will depend on which of the two factors predominates. In the experiments reported here, one of the factors may be selectively removed by a third substance which by itself has no action on surface denaturation.

These data indicate that certain agents enhance interfacial denaturation because they weaken side chain linkages in protein molecules. No appreciable increase in interfacial denaturation was observed if these agents were removed before exposing the proteins to interfacial forces. The increased denaturation, therefore, results from the simultaneous action of surface forces with the chemical agents.<sup>17</sup>

If the proposed mechanism of action of stilbamidine is correct (see discussion on TMV), then the action of Na-ribonucleate is to remove stilbamidine from the protein molecules. On removal of the diamidine, the linkages which were previously broken by stilbamidine must have the capacity of reforming in such a way that the protein, even though once treated with stilbamidine, has approximately the same resistance to surface denaturing forces it possessed before stilbamidine was applied.

Presumably, not many side chain linkages are broken by stilbamidine, but these may be sufficiently important so that, on superimposing surface forces, the stilbamidine-treated molecule becomes considerably unfolded. In this connection, Crammer and Neuberger<sup>18</sup> pointed out that phenolic groups may be largely, but not exclusively responsible for the configuration of native ovalbumin molecules. On the basis of the toxicity of BPA + stilbamidine, to kidney tubules in tissue culture, Kopac<sup>17</sup> has suggested that phenolic groups may be released by the action of stilbamidine on the BPA molecule.

#### *Interpretation and Significance of the Spontaneous Devaux Effect*

An explanation of the Devaux effect as demonstrated in the *Asterias* oocyte may also explain the way in which proteins are organized in living cells. The surface chemical properties of cytoplasmic proteins can be summarized in three statements: (1) little, if any, surface denaturation occurs at experimentally introduced oil-water interfaces in contact with cytoplasm; (2) maximum surface denaturation, at appropriate oil-water interfaces, can be demonstrated when the oils are administered at the instant of cytolysis; and (3) the surface denaturation of cytoplasmic proteins diminishes with length of the post-cytolytic period.

The following scheme, obviously over-simplified, was devised to account for the Devaux effect and other surface chemical properties of cytoplasmic proteins. The interpretations are based largely on the information obtained from a study of model systems. The modification in surface denaturation of albumin and other proteins by certain aromatic diamidines, for example, indicates the important role of low molecular weight substances in maintaining the stability of protein molecules. Furthermore, these diamidines react with nucleic acid and with nucleotides to form inactive complexes so that their action on surface denaturation may be completely neutralized.

In the scheme outlined in FIGURE 15, the cytoplasm, consisting of matrix as well as granules, cytolyzes by disintegrating into a variety of complex substances of which two are specifically indicated. One of these is a nucleoprotein complex,  $N_aP_a^{nxy}$ , that contains, *inter alia*, nucleic acid,  $N_a$ , a complex protein of the type exemplified by viruses,  $P_a^n$ , a surface denaturing

factor,  $x$ , and an anti-surface denaturing factor,  $y$ . The other substance is also a nucleoprotein,  $N_bP_b$ , but in this complex, the protein moiety is probably of low molecular weight, similar to the protamines or histones. Some of the  $N_bP_b$  complexes may even be the split products of the  $N_aP_a^{nxy}$  complexes.

The aggregation of the complexes,  $N_aP_a^{nxy}$  and  $N_bP_b$ , to form an ultra-structure of the cytoplasmic matrix may be of two types: (1) spherical aggregates, the protein complexes rich in phospholipids and ribonucleic acids, and (2) linear aggregates, the protein complexes essentially rich in ribonucleic acids. Of the two, the spherical aggregates appear to have a greater stability since these are the sub-microscopic particles that can be

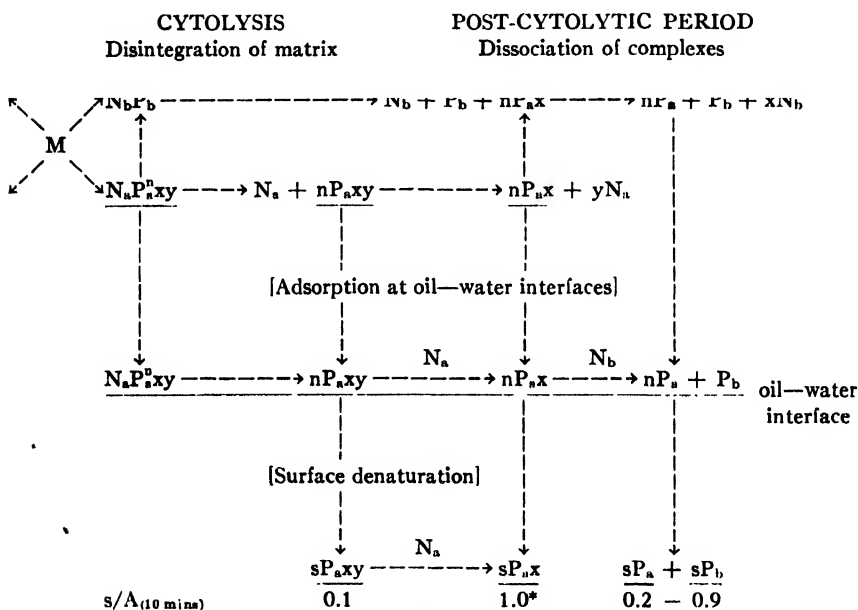


FIGURE 15. Schematic disintegration of cytoplasmic matrix into labile complexes and relation to Devaux effect. The complexes,  $sP_a^{nxy}$ ,  $sP_a^{nxy}$ ,  $sP_a$ , and  $sP_b$  refer to surface denatured proteins. The other symbols are discussed in text.

\*  $S/A = 1.0$  in less than 1 minute, the spontaneous Devaux effect.

isolated, from various cells, under appropriate conditions, by centrifugal fractionation (Claude<sup>19</sup>; Brachet<sup>23</sup>). These aggregates or particles also possess a variety of enzymatic activities (Brachet<sup>23</sup>).

The linear aggregates may resemble the insulin fibrils or filaments described by Waugh,<sup>24</sup> except in cytoplasm such filaments may consist mainly of high molecular weight protein complexes of the virus type. Furthermore, these filaments may be much more labile than the insulin filaments, since this type of aggregate has not, as yet, been isolated from cytoplasmic residue.

At present, it is assumed that both types of complexes can be arranged in linear as well as spherical aggregation. On cytolysis, the linear aggregates

may be the first to disintegrate and to release the  $N_aP_a^*xy$  and  $N_bP_b$  complexes. According to Brachet, the low molecular weight fraction (supernate) of preparations from which the cytoplasmic sub-microscopic particles can be isolated, by centrifugal fractionation, contains a great deal of ribonucleic acid, much of which could be in the form of  $N_bP_b$  complexes. The spherical aggregates probably disintegrate during the early post-cytolytic periods, unless the cytoplasmic residue is treated to prevent such disintegration.<sup>19, 22</sup>

The nucleoprotein complex,  $N_bP_b$ , is assumed to be more stable than the  $N_aP_a^*xy$  complex, and its dissociation occurs after the onset of cytolysis, the probable half-life being of several minutes' duration. The other complex, on the other hand, may dissociate at the onset of cytolysis with a probable half-life period of seconds.

The complex,  $N_aP_a^*xy$ , may dissociate progressively to form two fragments, nucleic acid and the protein components with adhering  $x$  and  $y$  substances. Along with this dissociation, the high molecular weight protein must dissociate into smaller molecules,  $nP_a$ . As the nucleic acid becomes released from the protein moiety, it can now attach to itself, the  $y$  or anti-surface denaturing factor.

There is no reason for doubting that the nucleoprotein complex,  $N_aP_a^*xy$ , becomes adsorbed at appropriate oil-water interfaces. It is doubtful, however, whether this complex undergoes surface denaturation owing to the macromolecular state of the protein and to the presence of the anti-surface denaturing factor,  $y$ .

Similarly, the complex,  $P_a^*xy$ , may be expected to become adsorbed at oil-water interfaces. Judging from the experimental data (see FIGURE 9,C and 9,D), no appreciable surface denaturation of this complex occurs. Although the low molecular weight proteins are more susceptible to surface denaturation, the presence of  $y$  suppresses this reaction.

With the appreciable release of nucleic acid,  $N_a$ , and subsequent binding of  $y$  to form an inactive  $yN_a$  complex, the remaining residue,  $nP_ax$ , is highly susceptible to surface denaturation. Here the proteins are of low molecular weight and in the presence of a surface denaturing factor,  $x$ . At this point, a spontaneous Devaux effect can be obtained at appropriate oil-water interfaces.

Thus, the spontaneous Devaux effect is made possible by the dissociation of complex nucleoproteins into nucleic acid and lower molecular weight protein molecules. Moreover, the rapid unfolding of adsorbed proteins at oil-water interfaces is enhanced by the removal of factors that normally maintain structural stability of the proteins by protecting them against surface and other denaturing forces.

Both high and low molecular weight proteins may be weakened or strengthened against surface denaturation by appropriate chemical agents. For example, stilbamidine enhances the surface denaturation of all proteins, so far tested. On the other hand, several agents (phenamidine, bis-amidinomethylbenzyl, protamine, *etc.*) can protect macromolecular nucleoproteins (TMV), cytoplasmic proteins (isolated from sea-urchin eggs), and simple proteins against surface denaturation. It should be noted that 1,2-di-*p*-anisylethylamine + stilbamidine inhibits the surface denaturation

of liver nucleoprotein, more so than does either substance alone<sup>14</sup>. This example approaches the situation postulated in the  $nPrx$  complex.

The granular components of the *Asterias* oocyte, namely, mitochondria, yolk, and others, are not essential factors in the production of spontaneous Devaux effects.\* For example, the most rapid development of the Devaux effect occurs on injecting an indicator oil drop into the optically homogeneous, germinal vesicle.

Significant amounts of surface denaturation can occur only if the nucleoprotein complexes dissociate with loss of the protecting  $y$ -factor. The protecting factor may either diffuse away, or else it may become bound to other agents released on cytolysis. On the basis of model experiments, the binding of the  $y$ -factor by nucleic acids seems the more probable.

The experimental data summarized, in FIGURE 14, suggest one possible mechanism. The surface denaturation of bovine albumin (5 mg./ml.) was almost completely inhibited by protamine (1 mg./ml.). On adding Na ribonucleate, much of the protamine was removed by formation of a colloidally dispersed, protamine-nucleate and, consequently, the albumin then became susceptible to surface denaturation. Thus, the removal of a protecting agent increased the susceptibility of the protein to surface denaturation.

The third statement suggests that cytoplasmic proteins have been reduced to smaller proteins, ordinarily the more stable molecules. The rapid development of spontaneous Devaux effects at the instant of cytolysis can best be explained by assuming the breakdown of unprotected macromolecular complexes, with a release of agents that enhance surface denaturation. Thus, along with the binding of  $y$  by nucleic acid, the activity of  $x$  now becomes possible. The action of  $x$ , a surface denaturing factor, would resemble the action of stilbamidine.

As the post-cytolytic periods are prolonged, the surface denaturation of the protein residue is less pronounced. This can be shown by the progressively lower  $s/A$  values, as measured by drop-retraction methods (see FIGURE 9, A and B). Such an effect might be expected if the  $x$  substances either diffused away or became bound by other products of cytolysis. For example, if stilbamidine is bound by nucleic acid, the proteins previously exposed to stilbamidine become less susceptible to surface denaturation.

The dissociation of the simpler  $N_bP_b$  complexes becomes of significance during the post-cytolytic period. The breakdown of this complex releases additional nucleic acid,  $N_b$ , which can then bind the surface denaturing factor,  $x$ , through the formation of the  $xN_b$  complex. The protein moiety,  $P_b$ , released on dissociation of the nucleoprotein may become adsorbed and surface denatured if of the histone, or more complex, type. If, on the other hand, the protein is of the protamine type, the adsorption and subsequent surface denaturation of the  $P_a$  type proteins may be inhibited. This would lead to lower  $s/A$  values as shown by the measurements of this quantity during late post-cytolytic periods.

\* Under appropriate conditions, the disintegration of the granular components obtained from living cells may be prevented.<sup>10,21</sup>

The mechanism of inhibition by the protamine types of proteins is perhaps largely concerned with the interface rather than with the protein. Protamine undergoes some surface denaturation, but such unfolded molecules do not remain trapped at the interface except in special instances (Kopac<sup>17</sup>). Instead, they become displaced with increasing surface pressures, with the result that the measured values of  $s/A$  become exceedingly low (see action of protamine on surface denaturation of bovine albumin). Furthermore, such proteins may prevent the adsorption of those protein molecules that ordinarily would unfold and become trapped at the interface.

Thus, the protective action on proteins by anti-surface denaturing agents may be produced by two separate mechanisms. On the one hand, the agent may fortify strategic side chain linkages in the protein molecule, thereby protecting it against the action of surface forces. On the other hand, the agent may prevent the susceptible protein from reaching the interface. In either instance, the surface denaturation of the protein would be appreciably reduced.

#### *Other Properties of Cytoplasmic Proteins*

Other data also indicate that cytoplasmic proteins in intact cells are different from the isolated proteins. Pflüger<sup>25</sup> differentiated between the dead, or storage proteins, and the live, cytoplasmic proteins which he termed *lebendiges Eiweiss*.

Pollack<sup>26</sup> reported that solutions of picric acid when injected into amebas produced a coagulating action only if a local injury was produced at the site of injection. The same concentrations of picric acid, however, readily coagulated proteins, *in vitro*. These data might indicate that the proteins in intact amebas cannot be coagulated by picric acid, unless some cytolysis has been induced by the microinjection. Needham<sup>27</sup> has commented on the possible significance of Pollack's experiments, but he also cautioned that the neutralization of picric acid by cytoplasmic components might also be a factor. As mentioned earlier, the injection of trichloracetate into intact *Asterias* oocytes produced immediate fixation without the appearance of typical cytolytic reactions.

Perhaps the most suggestive data that cytoplasmic proteins of living cells differ from the isolated proteins have been presented by Vlès and Gex.<sup>28</sup> The ultra-violet spectrophotometric curves of intact sea-urchin eggs were shown by Vlès and Gex to differ strikingly from the absorption curves of eggs cytolyzed by hypotonic solutions or by crushing. The absorption curves of the cytolyzed eggs resembled the absorption curve of a solution of ovalbumin. These differences in absorption spectra between living and cytolyzed sea-urchin eggs suggest that cytoplasmic proteins are structurally different from those recovered in cytolyzed eggs.

It is well known that living cells are not attacked by pepsin or by trypsin, whereas dead cells are rapidly digested. Fermi<sup>29</sup> concluded that the configuration of the protein molecule in the living cells was different from that after death of the cell and that the "living molecule" could not be attacked by the enzyme.



Northrop<sup>30</sup> showed that neither pepsin nor trypsin penetrated living cells. The injection of large volumes of active trypsin solutions into amebas initiated rapid streaming and the injected solution collected into a spherical, granule-containing blister. Though the blister might be pinched off, the ameba became motionless and disintegrated within a few hours.

The microinjection experiments suggested but did not prove that living cells are susceptible to trypsin hydrolysis providing the enzyme reaches the cytoplasm (Northrop<sup>30</sup>). Northrop<sup>31</sup> later admitted that the microinjection data "were not entirely convincing owing to the difficulty of performing the operation without injury to the cells."

In view of the surface chemical behavior of cytoplasmic proteins, before and after cytolysis, the resistance of living cells to proteolytic enzymes is to be expected, since these proteins seem to be protected against certain denaturing forces as long as the cells remain intact. The resistance of native protein molecules to proteolytic enzymes is fairly well established (Neurath, Greenstein, Putnam, and Erickson<sup>32</sup>). Furthermore, the virus nucleoproteins are not attacked by proteolytic enzymes to any appreciable extent (Pirie<sup>33</sup>).

Accordingly, Fermi's concept that 'living molecules' are responsible for the resistance of living cells to proteolytic enzymes may be reinterpreted on the basis that cytoplasmic proteins in the intact cell cannot be readily denatured and that unless denaturation does occur, proteolytic activity will be significantly blocked.

It is obvious that the problems of cytoplasmic proteins cannot be entirely answered with available information. We do have certain clues, however, and micro-surface chemical methods offer one way in which additional information can be obtained.

The study of surface chemical behavior of simple protein preparations, at various oil-water interfaces, together with modifications of these properties by various chemical agents, is a step in this direction.

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## SALIVARY GLAND CHROMOSOMES

By Ethel Glancy D'Angelo

Queens College, Flushing, New York

The cytogenetic implications of the structure of the salivary gland chromosomes of dipteran larvae, together with their large size and visibility *in vivo*, have prompted many investigators to use these chromosomes for studies on structure and physical properties. The characteristic appearance of the salivary chromosome, with its distinctive pattern of chromatic bands and achromatic interband regions, is now well known. It is in regard to the finer details of structure, however, that much controversy exists. At least five hypotheses are current regarding the structure of these giant chromosomes: (1) the alveolar concept proposed originally by Metz and Lawrence,<sup>1</sup> who believe that the striations seen in smear preparations are merely the result of drawing out the walls of alveoli and are not chromonemata, which to them are submicroscopic; (2) the polytene or multiple thread concept of Bauer<sup>2</sup> and of Painter and Griffin,<sup>3</sup> who maintain, respectively, that the visible striations are single chromonemata or bundles of chromonemata; (3) the view of Kodani,<sup>4</sup> based on alkali-urea treatment of the chromosomes, that there are only four chromonemata; (4) that of Ris and Crouse,<sup>5</sup> to the effect that the salivary chromosome consists of helically coiled chromonemata and that the alleged chromomeres are misinterpretations of these coils; and, finally, (5) that of Hinton,<sup>6</sup> that the bands are isolated groups of genic material separated from neighboring bands only by space, perhaps filled with nucleoplasm, and surrounded by a sheath.

There is generally more accord among investigators regarding the physical properties of these chromosomes. The findings of Vonwiller and Audova,<sup>7</sup> Barigozzi,<sup>8</sup> Stefanelli,<sup>9</sup> Pfeiffer,<sup>10</sup> and Buck<sup>11</sup> have indicated the salivary chromosomes to be tough, viscid, elastic gels. These observations are in essential agreement with those originally made by Chambers and Sands<sup>12</sup> on the chromosomes of *Tradescantia* and those by Chambers<sup>13</sup> on *Dissosteira* spermatocytes. These studies were extended by Buck,<sup>11</sup> who reported more detailed findings on the extensibility and elasticity of osmic vapor-treated salivary chromosomes. The experiments of Duryee<sup>14, 15</sup> on amphibian chromosomes indicated clearly how important it is that proper precaution be taken, inasmuch as the physical properties of the chromosomes could be modified extensively by the presence of either torn cytoplasm or calcium ions.

The present report will describe various observations made on the structure and physical properties of the salivary chromosome in the fresh state as determined by the technique of micromanipulation. The genus chosen for study was *Chironomus*, which is characterized by a flat salivary gland, one cell layer thick, in which the four chromosomes are plainly visible. The chromosomes are of especially large size, the largest being about 20  $\mu$  in diameter and approximately 150  $\mu$  in length. These factors are obviously important in facilitating micromanipulation.

It must be admitted freely at the outset that no criterion exists which can be used to decide indisputably whether the salivary chromosomes are living or not, inasmuch as these chromosomes never undergo mitosis and ultimately undergo autolysis in the pupal stage. It is felt, however, that the most rigorous approach in this type of study is one which must involve maintenance of the general appearance and properties of the chromosomes characterizing those in the freshly dissected gland. Precautionary methods, shortly to be described, were established, therefore, to preserve this fresh state through the period of observation. The experiments performed fall into two categories, those dealing with chromosomes in the intact cell, and those on isolated chromosomes.

### *The Intact Cell*

In studies on the chromosomes in the intact cell, the salivary gland was quickly dissected from the larva into isotonic Ringer's solution and transferred immediately to a fresh drop of Ringer's on a coverslip, which was then mounted on the moist chamber of the Chambers micromanipulator. Two precautions were necessary in such a dissection: (1) to prevent the gland from coming in contact with torn larval tissues; and (2) to remove the gland immediately from the original medium, which becomes progressively more acid in the presence of injured tissues. When these precautions were taken, the chromosomes were always distinctly visible, with relatively little space between them. The bands were generally well defined and were either homogeneous or beaded, the beads going through the chromosome. Occasionally, delicate longitudinal striations were visible in the interband regions, although these structures were usually homogeneous.

Observations and micromanipulation experiments were routinely made with a Leitz microscope using a 1.8 mm. oil immersion objective, N.A. 1.25, and a 10 X ocular. A micrometer ocular was used for measurements.

Tearing the cytoplasm with a microneedle caused marked changes in both the nucleus and the chromosomes. The nucleus swelled about 50 per cent in diameter, and the chromosomes became dark, shrunken, and so sticky as to be unmanageable. These changes appeared to be in part, at least, the result of "acid of injury," since they could be duplicated by immersion of the gland in an acidic medium. Puncturing the cell with a large microneedle produced effects essentially similar to those caused by tearing the cytoplasm. By using a graded series of microneedles, however, it was found that a microneedle of less than 1  $\mu$  toward the tip produced no visible change in the nucleus or chromosomes. Consequently, in all my experiments these fine microneedles were used.

*Stickiness.* The question of whether or not the chromosomes are sticky has often been raised in the literature. Practically all investigators who have manipulated the isolated chromosomes report them to be highly sticky. The fact remains that chromosomes within the intact nucleus do not appear to adhere to each other. The following experiment was done to investigate this point.

Two microneedles were inserted into the nucleus and manipulated in such

a way as to force two chromosomes together. Chromosomes approximated in this manner never stuck together. They appeared always to be separated by a narrow region of hyaline material representing the jelly-like nuclear matrix.<sup>16</sup> Oil drops or carbon particles which were injected into the nucleus as close as possible to a chromosome failed to adhere to the chromosome, as might be expected if the chromosomes are actually non-sticky. On the other hand, when the cell was torn, the chromosomes readily stuck together and could be separated only by pulling out thick, viscid strands. Injected oil drops or carbon particles now readily adhered to these chromosomes. It would appear, thus, that chromosomes in the intact nucleus become sticky by experimental treatment and it is likely that this phenomenon involves liquefaction of the nuclear matrix which normally keeps the chromosomes separated.

*Consistency.* Experiments on the consistency of the chromosomes showed them to be almost fluid in the intact uninjured cell. They were easily deformable by even slight pressure from the microneedle. Of course, any measurement on the consistency of chromosomes must of necessity be relative, and at best only rough estimates can be made. The relatively low consistency of the intact chromosome, as compared to other nuclear structures and cytoplasm, could be demonstrated by the injection of oil drops. When an oil drop was injected directly into the jelly nuclear matrix, a rounded depression was produced in the nearest chromosome, indicating that the consistency of the chromosome is less than that of the nuclear matrix. The consistency of the chromosome, on the other hand, appears to be greater than that of the nucleolus. This was demonstrated by separating the nucleolus from the small fourth chromosome (to which it is normally attached), whereupon the nucleolus became spherical, and then approximating the free spherical nucleolus to a chromosome. When this was done, the nucleolus assumed the contours of the chromosome, thus demonstrating its lower consistency. Differences in consistency could be demonstrated within the chromosome itself. Rough estimates of the relative consistency of the band and interband regions were made by determining the rate at which a micro-puncture would disappear. A puncture made in the nucleolus closed over most rapidly, one in the interband regions less so, and one made in a thick band most slowly of all. It seems from these experiments, then, that the consistency of the chromosome as a whole lies between that of the nucleolus and the jelly-like nuclear matrix, and that within the chromosome the bands have a greater consistency than the interbands.

*Extensibility and Elasticity.* Attempts were made to determine the extensibility and elasticity of the chromosome within the nucleus by inserting two microneedles into a short section of a chromosome, then slowly drawing them apart. When this was done, the region under tension became fainter as the chromosome was stretched. The interband regions stretched more readily than the bands and consequently suffered a greater decrease in diameter. The thick bands stretched more at their margins than in the center. With increased stretching, these thick bands often separated into several narrow bands, thus demonstrating their compound nature. Faint longitudinal striations, which followed the lines of tension, generally

appeared in the interband regions when a chromosome was stretched more than twice its length. They did not appear to be identical with the delicate longitudinal striations occasionally seen in unstretched fresh chromosomes. On release of tension, the striations disappeared and the chromosome, even after being stretched up to five times its length, returned to its original condition. It was not found possible to stretch a given region more than this without tearing the nuclear membrane. More extensive experiments will be considered in the section on the isolated chromosomes.

*The Chromosome Membrane.* There has been much controversy as to whether or not the chromosome possesses a membrane or sheath. Painter originally thought a membrane was present, but more recently<sup>17</sup> has reversed his opinion. Metz<sup>18</sup> believes a sheath to be necessary on theoretical grounds. The experimental evidence which follows is in accord with the presence of a membrane.

A microneedle was inserted into a margin of a chromosome and then gently withdrawn a short distance. Attached to the needle could be seen a delicate membrane-like material (FIGURE 1,a).\*

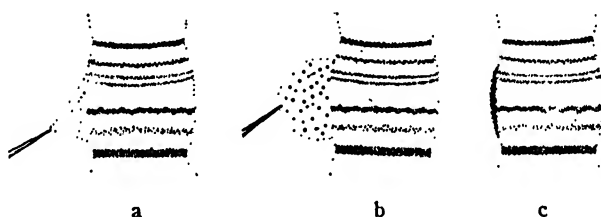


FIGURE 1. (a) Lifting by a microneedle of a membrane-like material from a portion of a chromosome within the intact cell. (b) Micro-injection of a carbon suspension into the area subjacent to the lifted membrane shown in (a). (c) Concentration of the carbon particles into a narrow zone as a result of gradual contraction of the membrane.

an aqueous suspension of carbon particles. The material suspected of being membranous in nature became distended (FIGURE 1,b). As the fluid was dissipated, the carbon particles became concentrated close to the chromosome (FIGURE 1,c). When larger amounts of fluid were used, the membranous material would suddenly burst, with the carbon particles scattering into the nuclear matrix. The experiment was repeated by injecting an oil drop instead of carbon particles. In this instance, the oil drop became attached to the chromosome by the membrane-like material previously described and movements of either one with the microneedle would carry along the other structure.

The effects of injecting water or salt solutions (0.01–1.0 M NaCl, KCl, and  $\text{CaCl}_2$ ) directly into a chromosome are indicated in FIGURE 2. In each instance, the bands disappeared in the region injected as the site gradually swelled, with the swelling eventually becoming localized by persisting heavy bands. If the swelling exceeded about three times the diameter of the chromosome, then the region would suddenly burst, indicating an elastic membrane whose limits had been exceeded. It seemed particularly significant

\* This figure and all subsequent ones have been previously published.<sup>21</sup>

that the bands would, in a short time, reappear in their original positions, but the distinct outer boundary of the chromosome was lacking in the injected region. When the injected solution was calcium chloride, a granulation appeared in the nuclear matrix around the injected zone, but this did not occur with the other solutions. It would seem from these experiments that the salivary chromosome possesses a delicate elastic membrane, and that there must be structural continuity between the bands and the interband regions, since they maintain their orientation even in the absence of the membrane.

*The State of the Chromosomes in Nuclei Rendered Optically Homogeneous by Experimental Treatment.* The reappearance of the bands in the experiments just described suggested the possibility that in optically homogeneous nuclei the chromosomes, though not visible, are really present. Hyaline nuclei were not found in the *Chironomus* glands examined, but they could be readily made so by immersing the gland in hypotonic or alkaline Ringer's solution.

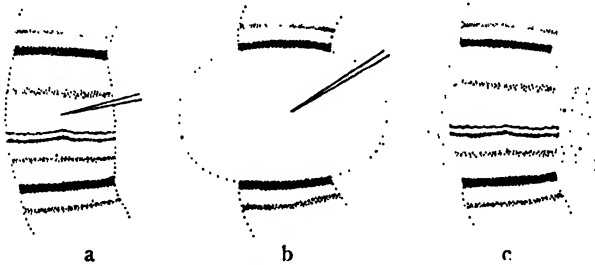


FIGURE 2. (a) Micropipette inserted into an interband region of a portion of a chromosome showing the initial stage of injection. (b) Localized swelling and loss of structure in the injected portion of the chromosome. (c) The appearance of the same chromosome after injection of sufficient  $\text{CaCl}_2$  to rupture the injected region. The bands have reappeared in their original positions, but the distinct boundary shown in (a) is lacking. Note the granular region of the nuclear matrix.

Microneedles were moved back and forth in such hyaline nuclei in an effort to detect some regional resistance which might indicate the presence of chromosomes, but there was none. Stefanelli<sup>9</sup> made the same observation and concluded that the chromosomes must therefore be completely dispersed. Such an experiment is not conclusive, however. To test this further, the following experiment was done. Carbon particles were introduced into both normal and hyaline nuclei. The carbon particles in normal nuclei aligned themselves in such a fashion as to outline the contours of the chromosomes. When the gland so injected was subsequently immersed in hypotonic or alkaline Ringer's solution, the chromosomes swelled and disappeared, but the carbon particles maintained their positions, outlining the ghost chromosome cylinders (FIGURE 3). Similar results were obtained in nuclei which were first made hyaline, then injected with the carbon suspension. In both cases, the chromosomes reappeared in the expected positions when the glands were placed in Ringer's solution. These results suggest that the chromosomes do not become dispersed in optically homogeneous nuclei, but that they maintain their morphological integrity.

**Chromosome Structure.** Many experiments were made to determine the finer structure of the chromosomes. As noted previously, the detailed structure was not generally visible in the fresh chromosome, although the bands were occasionally beaded and the interband regions occasionally showed delicate longitudinal striations. These striations were visible at all levels in the chromosome and appeared to be connected to the beads in the bands. An alveolar appearance was never observed in the fresh chromosome, but the alveolar condition was seen often in aceto-carmine or Feulgen preparations.

Chromosomes were treated with various reagents in an attempt to dissolve differentially the chromosome so that its basic structure would be more clearly revealed. Results of this type are valuable but must be interpreted with caution, since it was found possible by experimental treatment to cause the chromosome to appear fibrous (with alkali<sup>17, 19</sup> or alkaline urea<sup>4</sup>), alveolar (with NaCl,<sup>17</sup> KCl, or CaCl<sub>2</sub>, the latter only if injected), granular (with

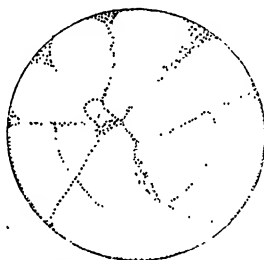


FIGURE 3. Alignment of carbon particles outlining the contiguous borders of adjacent chromosomes in an optically homogeneous nucleus. The nucleus was first injected with a carbon suspension, then hyalinized by immersion of the gland in hypotonic Ringer's solution.

NaCl,<sup>17</sup> KCl, acids, or detergents), or homogeneous (with organic solvents). Accordingly, recourse was had to other methods.

If chromonemata are really present, it should be possible to obtain evidence from micrurgical experiments. These were done as follows. Oil drops were injected into the interband regions of the chromosomes to determine whether the oil might possibly become elliptical in shape as a result of compression between alleged chromonemata. The injected oil drops were, however, always spherical. This would tend to support the alveolar contention of Metz and Lawrence<sup>1</sup> or Hinton's concept<sup>6</sup> of structureless interbands. But it does not necessarily invalidate those concepts which involve the presence of chromonemata, since it will be remembered that the chromosomes have a very low consistency and therefore existing chromonemata, if any, could easily be displaced.

Attempts were made to obtain evidence from lateral stretching experiments, since there was the possibility that any existing chromonemata might be separated in this way. Two microneedles were inserted into the margins



of a chromosome and then slowly pulled apart. It was observed that the band, being stretched, became beaded (FIGURE 4). This might be taken to indicate that the chromomeres were being separated from each other. (A similar beaded appearance was observed when the nucleus was injected with water or dilute salt solutions.) The interband regions showed no chromonemata, but tension lines were visible.

More clear-cut evidence was obtained in microdissection experiments. The chromosome membrane was first removed over a short distance. Then a microneedle was inserted close to the edge of the chromosome. It was found possible to remove delicate longitudinal fibrils in this manner, even though no striations were visible in the region being dissected. The fibrils thus partially isolated displayed nodes at intervals corresponding to the position of the bands (FIGURE 5). These nodes did not disappear when the fibrils were stretched, indicating that they are not merely gyres in a coil as

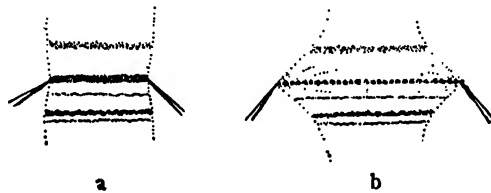


FIGURE 4. (a) Sketch of a portion of a chromosome prior to stretching. Two microneedles are inserted directly into a band. (b) Beaded appearance of the band when the chromosome is stretched laterally. Note tension lines in the interband regions.



FIGURE 5. A single fibril with a chromonema-like appearance being removed by a microneedle from a chromosome within the intact cell.

maintained by Ris and Crouse.<sup>5</sup> Similar fibrils could be obtained by injecting a small amount of fluid near the margin of a chromosome. When the injected fluid was calcium chloride, the fibrils were stiff and inelastic. On the other hand, fibrils isolated by the injection of water, NaCl, or KCl, were soft and highly extensible. It was not found possible to dissect any fibrils lying at right angles to the long axis of the chromosome, as should be equally possible in an alveolar structure. This evidence, as well as that shortly to be described from the isolated chromosomes, appears to support best the polytene concept of chromosome structure.

#### *Isolated Chromosomes*

The most important factors in the isolation of a single salivary gland chromosome are: (1) immediate removal of the chromosome from the torn

cell; and (2) quick transfer of the chromosome to a neutral calcium-free medium. In these isolation experiments, the injurious changes produced by the surrounding cytoplasm on the nucleus were minimized by rapidly tearing both the cytoplasm and the nuclear membrane. The operation was done with steel needles under a dissecting microscope. In this way, the chromosomes could be forced out of the nucleus by gentle pressure, quickly picked up by a lip pipette, and then transferred to the appropriate medium. No exhaustive attempts were made to find the ideal medium for the isolated chromosome, but of the various media tried, including hemolymph, Ringer's, paraffin oil, sucrose, albumen, Duryee's medium,<sup>15</sup> and 0.6% NaCl, the most satisfactory medium was one consisting of 0.09 M KCl, 0.06 M NaCl, buffered to pH 7.0 with phosphate. In this medium, the isolated chromosomes closely resembled those in the intact cell in both structure and physical properties for at least 5–10 minutes. All observations were made within this period.

It was found that relatively small changes in pH could cause extensive changes in the structure and properties of the isolated chromosomes. If the chromosomes were maintained at pH 7.6, reported by Chambers<sup>20</sup> to be the pH of the normal *Chironomus* nucleus, structure became faint, and the chromosomes became sticky and difficult to manipulate. Acidification of the medium to pH 6.5 caused the chromosomes to shrink, particularly in the interband regions, and structure to become more distinct. Such chromosomes were relatively stiff and inelastic. The consistency of the chromosomes increased progressively as the pH of the medium was lowered. The effect of calcium ions on the isolated chromosomes was generally like that of acidification.

The isolated chromosomes were especially satisfactory for stretching experiments. Both extensibility and elasticity were influenced considerably by the medium. Acidic solutions decreased both properties, whereas, in alkaline solutions, extensibility was greatly increased, the chromosomes becoming ductile and inelastic. In the neutral KCl-NaCl medium, chromosomes could be stretched about 10-fold and still recover their original length, although repeated stretching tended to decrease elasticity. In one case, a chromosome was stretched 25-fold, and when tension was released, it returned to slightly more than its original length (1.3x). This observation, perhaps, is significant in view of the established fact that long fibrous molecules possess unusual elasticity. When rupture occurred in one of these stretched chromosomes, the break invariably occurred in an interband region. Fibrillae were occasionally seen at the broken ends of the chromosomes. It should be pointed out that greatly stretched chromosomes developed a coarse fibrillar appearance and rapidly deteriorated. This fibrillar appearance was different from that observed in unstretched chromosomes in that these fibrils were relatively coarse and always followed the lines of tension.

I believe, however, that the following experiment indicates that true longitudinal fibrils do exist in the isolated chromosomes. It was possible to remove a whole chromosome directly from the nucleus by means of a very large micropipette. It was further possible to expel the chromosome from

the pipette into an appropriate medium. If the micropipette was of the correct caliber, the expelled chromosome would shred into numerous delicate longitudinal fibrillae which resembled the fibrils obtained by microdissection. The operation was only occasionally successful, for, if the micropipette was much larger than the chromosome, the chromosome would be expelled intact. The fact that this shredding of the chromosome into longitudinal fibrils can occur, however, is deemed significant, and this observation—together with the beading of the bands, the dissection of fibrils by microneedles, the occasional fibrillar appearance of untreated chromosomes, the presence of fibrillae at the broken ends of stretched chromosomes, and the return of the bands to their original positions after rupture of the chromosome membrane—tends to show the polytene nature of the salivary gland chromosome.

In summary, it may be concluded, from these micromanipulation studies, that: (1) the salivary gland chromosomes are highly extensible, elastic, soft, deformable bodies, and not viscid, tough gels as generally described by others; (2) the salivary gland chromosomes in optically homogeneous nuclei are not isolated or dispersed, but rather maintain their morphological integrity; (3) the salivary gland chromosome possesses a delicate elastic membrane; (4) the salivary gland chromosome is polytene in nature.

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### *Discussion*

IVOR CORNMAN (*The Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): Such superbly delicate probing into the chromosome is supplying us with the answers to questions which remained controversial the whole time that we were forced to rely upon indirect evidence of chromosome structure. Is the matrix which separates chromosomes, as made visible when the chromosomes are pushed together, to be visualized as a sheath around each chromosome? Can it be distinguished from the chromosomal membrane?

In a hyalinized nucleus, where the boundaries of the chromosomes are marked with carbon particles, can the chromosomes be moved by the micro-needle? If they are so fluid as to permit the needle to pass through them, how does a chromosome look when again rendered visible?

Can it be demonstrated that fibrils dissected from the giant chromosomes behave in a manner comparable to chromonemata, as for instance, spiralling in acid medium?

DOCTOR D'ANGELO: It may be shown that the chromosomes in salivary-gland nuclei are embedded in a jelly-like matrix by various micrurgical experiments. Carbon particles injected into the central region of the nucleus show no Brownian movement, whereas particles injected just under the nuclear membrane display Brownian movement. An oil drop injected into the central region of the nucleus remains in position, whereas one injected peripherally will tend to rise as would be expected in a fluid medium. The jelly-like matrix is thixotropic, as it may be liquified by mechanical agitation with the microneedle. The chromosome membrane is still demonstrable after liquefaction of the matrix has occurred.

Chromosomes outlined by carbon particles in hyaline nuclei may be moved about by microneedles, and are sufficiently fluid so that the needle may pass through them. When such a nucleus is returned to Ringer's solution the chromosomes appear distorted.

Further experiments must be performed before Dr. Cornman's last question can be answered.

# CHROMOSOMAL PHYSIOLOGY IN RELATION TO NUCLEAR STRUCTURE

By William R. Duryee

National Cancer Institute, Bethesda, Maryland, and Department of Terrestrial Magnetism,\*  
Carnegie Institution of Washington, Washington, D.C.

## *Statement of the Problem*

In every cell nucleus, there exists a structural as well as a physiological relationship between the chromosomes and the colloidal substrate in which they are embedded. Not only does the substrate maintain and protect the chromosomes in their relative positions, but it also transmits to them organic and inorganic molecules for synthesis. Even more important, the substrate collects and distributes the materials produced by the chromosomes. The basic significance for these processes in amphibian eggs of lateral loop synthesis was first presented by the author in 1937, followed by a general theory of the vertebrate chromosome (1941).

The periodic disappearance and reappearance of the lateral loop chromomere structures have led some, through fragmentary observations, to deny their existence. For example, Clark, Barnes, and Baylor (1942), using the electron microscope found no evidence for them. On the other hand, Boche and Anderson† saw them clearly and figured them in their electron photograph. Lateral branches of amphibian chromosomes were first described by Flemming (1882), while the loop structure was discovered independently by Born (1892), in amphibia, and Rückert, in sharks, in 1892. Studies by M. Gersch (1940), Painter (1940), Painter and Taylor (1942), Ris (1945), and Koltzoff (1938) on amphibian material have failed to show how chromosomes function. They disagree on the basic details of lateral loop structure and on results of Feulgen staining, as well as on the significance of nucleolar production. A more serious error has recently been made by P. Makarov (1946), who claims that *Rana temporaria* ovarian chromosomes are experimentally created “*de novo*” by fixatives penetrating the “homogeneous nuclear colloid.” He did not attempt to account for constant chromosome numbers or specifically recognizable chromosome pairs. With the exception of Gersch’s paper, which concentrated mainly on chemical tests of nucleoli, there is no adequate work on the physiology of animal chromosomes. Dodson (1948) has studied the Feulgen staining of amphibian chromosomes and is in general agreement with the morphological results reported here.

The main purpose of this paper is to show experimentally how chromosomal products are liberated from the chromosomes within the cell nucleus and transmitted to the cytoplasm. A secondary purpose is to present a new technique that is based on photographic records of fresh material under precisely controlled conditions. The older techniques of fixation and staining have led to no new point of view. Precipitation of colloidal proteins with harsh reagents, followed by long extraction in fat solvents and exposure

\* This work was done at the Marine Biological Laboratory, Woods Hole, Mass., and at New York University in the laboratory of Dr. Robert Chambers, to whom grateful acknowledgment is made herewith.

† See review by G. A. Morton.

to hot wax and aniline dyes, cannot be considered a physiological method. From a detailed study of alternately mounted sections of testis and ovary fixed in Zenker, Carnoy, Bouin, Aoyama, and other fluids, it is concluded that, while the spermatocyte material gives the ideal conventional but doubtless erroneous pictures, fixed ovocyte details differ from the normal *in every respect*. Moreover, an appreciation of correct geometry is essential to the study of the nucleus, and this cannot be obtained from dehydrated and sectioned material. One must always bear in mind that living processes can only take place in water-rich colloids (*cf.* Duryee 1938). The conclusions presented in this paper apply equally to both frog and salamander material except where specific differences are noted.

A method whereby the above difficulties could be circumvented was introduced by the author in 1936 for studying the relatively large ovocyte nuclei of aquatic vertebrates. It consisted essentially of slitting the ovarian egg to extrude the nucleus into a modified calcium-free Ringer solution.\* When washed immediately from adherent yolk and cytoplasm, the isolated nucleus clearly exhibited its chromosomes, nucleoli, and other constituents in their three-dimensional relationships. The nuclear membrane could then be dissected by hand, using glass needles, thus freeing the inner colloid gels with their 13 pairs of chromosomes and the multiple nucleoli, all of which were easily available for further study with reagents or for micromanipulation.

Isolated nuclei are not necessarily alive, nor are they to be considered dead, unless toxic reagents are added. The important fact is that they appeared normal in all essentials for periods up to one-half hour isolation, when recognizable degenerative changes may occur. The mild pH changes used in this technique for photography were almost completely reversible. PLATE 1 (A) gives a comparison of transparent eggs with their visible nuclei with one that has been isolated and floated into the field.

#### *Normal Nuclear Growth Cycle*

FIGURE 1 has been prepared to show, diagrammatically, the chief stages of normal germinal vesicle development and to indicate the concurrent changes in chromosome structure. It is convenient to divide the nuclear history into six arbitrary stages. These are typical of the frog, but with minor amendments they also apply to urodeles. Stage 1 is the smallest follicle in which chromosomes can be seen in the intact and transparent living egg, but without clear definition. In Stage 2, the chromosome pairs are now barely visible embedded in a nucleoplasmic gel, when the medium is slightly acidified below pH 5.8. Egg diameters are less than 200  $\mu$ . Comparison should be made with PLATE 1 (A). By Stage 3, where the eggs are from 200 to 500  $\mu$  in diameter, a few more details of nuclear structure can be observed through the surrounding transparent theca cells. Lateral loop production has now begun. A zone of large irregular nucleoli, prominent just beneath the nuclear membrane, characterizes this stage. Shadows of

\* Nuclear or N-Medium is made up of NaCl 0.66 gm., KCl 0.014 gm. in 100 cc. of Pyrex distilled H<sub>2</sub>O. This medium should not be buffered, except in cases where nuclear swelling is troublesome. In rare cases with small nuclei, it may be helpful to reduce the pH to 5.9 with KH<sub>2</sub>PO<sub>4</sub>.

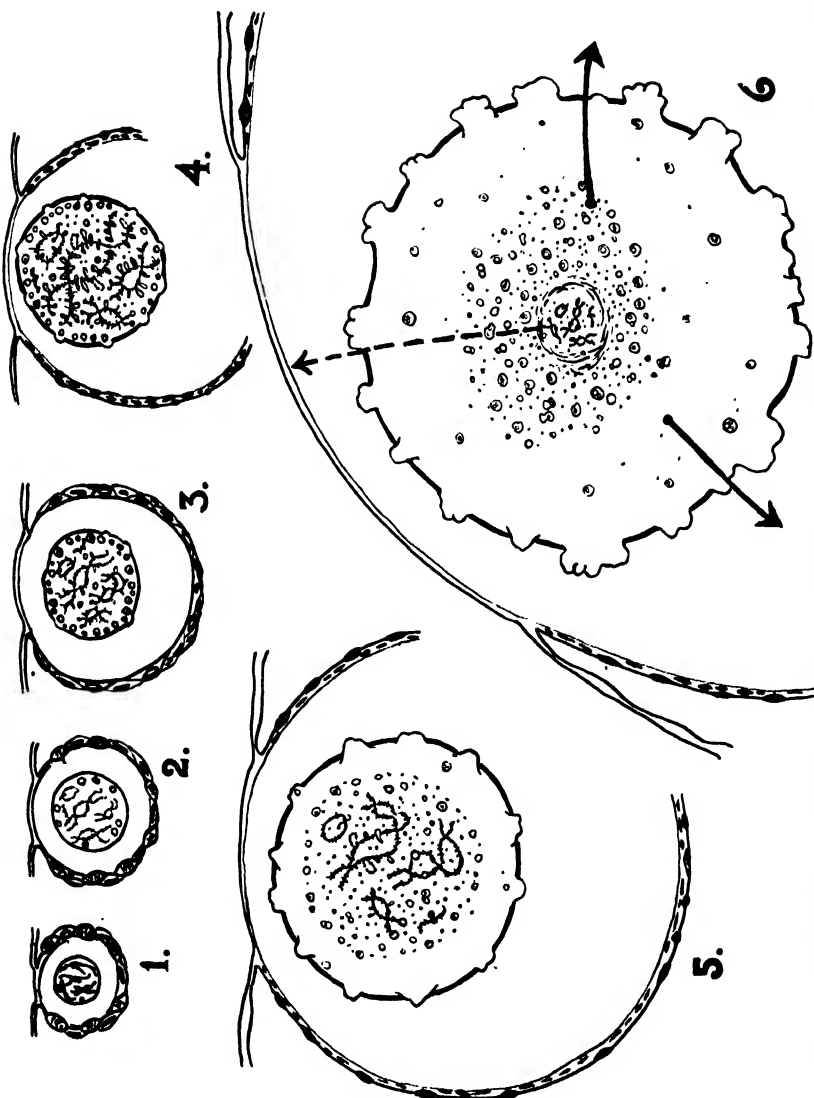


FIGURE 1. Schematic diagram of nuclear growth stages during the later development of frog eggs. Heavy arrows indicate mixing of nuclear material in cytoplasm after germinal vesicle membrane breakdown. Dotted arrow indicates migration of central chromosomal mass toward the animal pole to become the 1st polar body maturation spindle.

the paired chromosomes are barely visible under ordinary light, but are clearer with the phase contrast objective or in less alkaline media. See PLATE 1 (B). In Stage 4, the development of a yellow-brown color and addition of yolk prevent internal observation. Consequently isolation and washing of the nuclei are necessary. Eggs in Stage 4 range from 500 to 750  $\mu$  in diameter. Here the chromosomes of *Triturus pyrrhogaster* reach their maximum length of over 700  $\mu$  and have the most developed lateral branches. Frog chromosomes of comparable stages are approximately 60 per cent as large as those of urodeles. It is of interest that these Stage 4 amphibian chromosomes are the largest yet known in any animal, being nearly three times the size of the so-called giant chromosomes of dipteran salivary glands.

Up through Stage 4, growth of the supporting colloid gel, which I have designated the *chromosome frame*, keeps pace with the increasing diameter of the nucleus. This period of growth is shown in the top four diagrams of FIGURE 1. Photographs of Stage 4 before and after staining appear in PLATES 1 (C) and 2 (C).

By Stage 5, the chromosome frame begins its slow process of contraction. Eggs have now reached approximately half their maximum normal size, having diameters ranging from 750 to 850  $\mu$ . The contraction is not at first evident, since the nucleus as a whole continues to increase in volume, but becomes marked in comparative measurements made on older cells. Concurrently, the chromosomes are shortened and have progressively fewer and smaller lateral loops. Major nucleolar production is continued, as shown by new nucleoli appearing near the center and by an overall increase in number up to several hundred. Sac-like protrusions, which were first described by Duryee in 1937, are beginning to project from the surface of the nuclear membrane. Since these are formed structures of the membrane, remaining without change even in torn isolated membrane sections, the sacs may be spoken of as "organelles." In some species, they persist during a year or more of ovarian existence.

Stage 6, which is the last depicted, shows the germinal vesicle at its maximum growth, when the egg diameter has reached 1.8 mm. The diagram should be compared with PLATE 1 (E), which shows a nucleus from a somewhat smaller egg. The former indicates that the chromosome frame (= Karyosome) has shrunk to less than 1/1000th of the total nuclear volume, and is now coated by a denser substance, which has the property of being coagulated by calcium into aster-like fibers. The chromosomes have shortened to 40  $\mu$  or less and have lost all large lateral loops. A striking feature of this stage is the central cloud of large polyphasic nucleoli, surrounded by a few peripheral ones. Mixed with the central nucleoli are a number of distinctly smaller hyaline bodies having diameters from 0.5 to 2.0  $\mu$ . These latter may be called *loop fragments*. Together with the larger nucleoli they are embedded in a physically separable gel concentric with the chromosome frame. The fact that chromosome frame substance is a morphological entity in the nucleus can be seen in PLATE 1 (D). Here the other supporting gels have been dissolved, allowing the chromosome frame to sink



to the inside of the nuclear membrane as a spherule. Its viscous nature is shown in PLATE 1 (F).

As has long been known, the history of the egg nucleus terminates with a process of dissolution of the membrane and a belated intermingling of

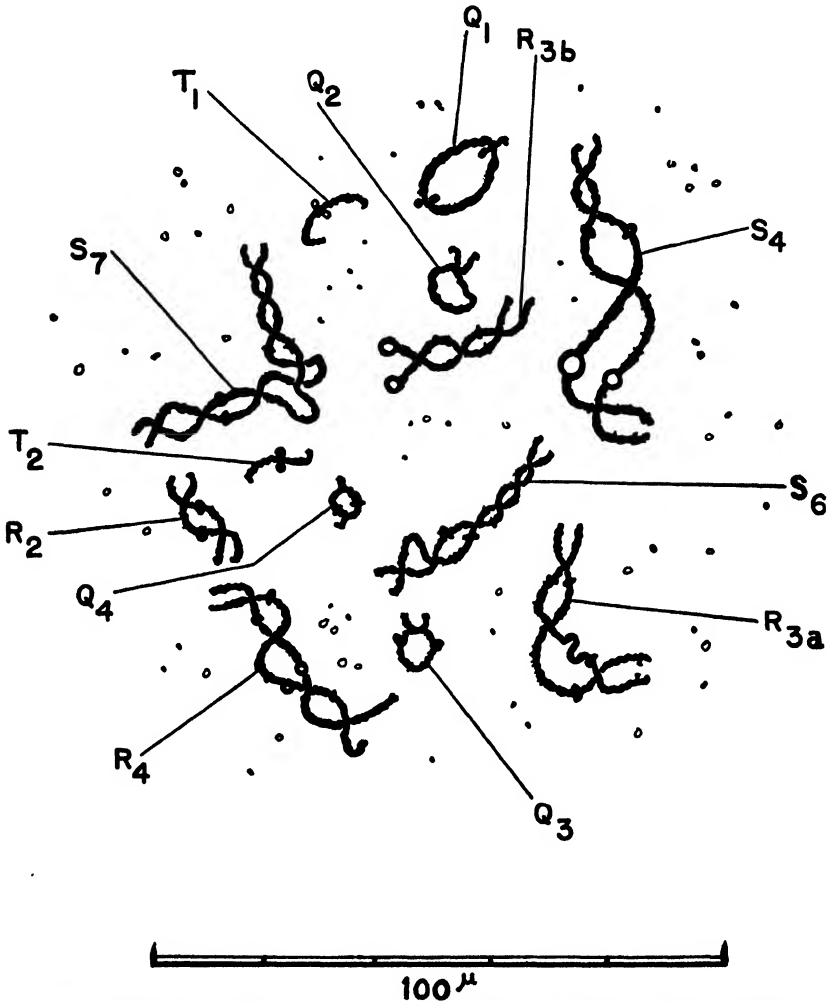


FIGURE 2. Camera lucida sketch of complete chromosome set in the germinal vesicle of a Stage 6 frog egg. Identifying letters have the following significance: *Q* for ring or "Q"-shaped pairs (4); *R* for intermediate pairs (4); *S* for long or super pairs (3); and *T* for short "T"-shaped pairs (2). Subscript numbers help the identification of the *R* and *S* group by indicating the number of chiasmata which is constant for a given chromosome pair. In the case of *T* and *Q* sets, they indicate merely decreasing size.

germinal vesicle contents with the egg cytoplasm. This process initiates a profound metabolic reaction which exhausts the cell unless fertilization shortly ensues. At the same time, the chromosome frame, having been set free, is converted into the 1st maturation spindle preparatory to extrusion of the polar bodies [*cf.* FIGURE 1(6)].

*Identification of Isolated Chromosomes*

FIGURE 2, a camera lucida sketch, shows the relative shapes and sizes of the 13 pairs of chromosomes in a Stage 6 nucleus of *Rana temporaria*. From comparison of egg nuclei in the following species, *R. temporaria*, *R. esculenta*, *R. pipiens*, *R. calesbiana*, and *R. clamitans*, it is clear that the numbers and individual characteristics of the chromosomal pairs are remarkably constant. This leads to an advantage for studies of chromosome physiology, because each individual pair can be precisely identified. For convenience, they have been designated with the letters *Q*, *R*, *S*, and *T*. Shown in FIGURE 2 are four ring or Q-shaped pairs identified by the letter *Q* and subscript numbers in order of decreasing size. Next come four medium pairs lettered *R* with numbers corresponding to the number of chiasmata. *S* designates the three longest or super pairs, again with subscripts referring to the number of chiasmata. The letter *T* identifies the two short T-shaped pairs. Sufficient data are not yet at hand to permit a similar chromosome identification system for the other groups of amphibia.

*Basic Chromosomal Structure*

The amphibian chromosome, because of its size, permits an analysis of structural elements not heretofore possible. Much of the confusion that exists regarding interpretation of stained and fixed material can be avoided by the study and photographic recording of freshly isolated nuclear components. The details are sharp and clear. In this section, the basic elements of chromosomal structure are presented, followed by the experimental and analytical data. First to be described will be those chromosomes without lateral loops.

FIGURE 3 illustrates the fact that both frog and salamander chromosomes have a similar structure. There is an apparently single chromonema along which compound granules and chromomeres of varying shape and size are firmly embedded or attached. Both pairs of chromosomes were in normal intra-nuclear position when sketched. The salamander pair, being under slight tension except for the left-hand end of one univalent, shows the general correspondence between homologous chromomeres. At certain places, however, a few of the visible structures do not appear to correspond. In chromosomes such as these, which have been treated with 0.2 M.  $\text{NaHCO}_3$  to remove the lateral loops and reveal the chromonemata, there is no evidence of uncoiling or breakage of longitudinal elements. Occasional loops may still persist after this mild treatment showing they are not part of the chromonemata. Photographs in PLATES 2 (A), 5 (C, D, E, and F) and 6 (A and B) illustrate the same details. The thicker oblong areas on these chromosomes are gelatinous or sometimes viscous coatings of the longitudinal chromonemata. Since this sheath-like coating is similar to that described for other types of plants and animals by various authors, e.g. Nebel (1939), as the chromosome "matrix," that term will be retained here. It is not so easy to homologize the terms "chromomere" or "chromiole" with the structures in FIGURE 3. By definition, a chromiole is the smallest visible particle,

constant in size, that remains embedded in a chromosome. Until the chemistry of these particles is better known, I prefer the term "chromosome granule" to refer to such particles  $1\ \mu$  in diameter or less. "Chromomere" is the name correctly applied to an individual discrete segment of somewhat larger size, together with its cluster of lateral branches described below. As far as can be seen in the accompanying photographs, there are seldom more than two granules per chromomere. However, it is possible that adjacent chromomeres may fuse, as shown in PLATES 5 (C) and 6 (B).

The second type of basic structure is that of the Stage 4 and 5 chromosomes with large lateral chromomere loops. FIGURE 4 is a diagram of the loop cluster arrangement in normal nuclei at different stages. This figure should be compared with PLATES 2 (B, C), 3 (E), and 4 (A, B, C). In the diagram at the left of FIGURE 4, several clusters of lateral loops (1) are shown

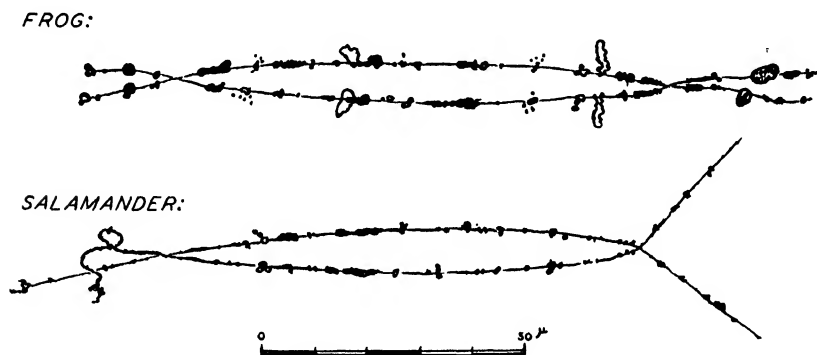


FIGURE 3. Camera lucida sketch of Stage 4 chromosomes. Short exposure to  $0.2M\ NaHCO_3$  followed by mild acidification has removed most of the lateral loops. Chromosome granules are attached to the paired chromonemata at homologous loci. Some matrix coating is present.

held together by a single chromonema ( $k$ ). Attention is called to two other important points, namely, that there are different numbers of loops per cluster, and that loops are not all of the same construction. At the right of the same figure is a diagram of a chromosome from a Stage 5 or early Stage 6 nucleus. Most of the large loops have disappeared, leaving an accumulation of loop fragments. The few that remain often show partly straightened broken branches, which in no way affect the chromonemata. The granules and the chromonema have, in certain areas, acquired coatings of matrix material ( $m$ ). This is a weak gelatinous coating not to be confused with the longitudinal thread.

#### *Chromosome Formulae*

By use of the letters shown in FIGURE 4 and explained in the previous paragraph, a convenient system of formulae has been made. The annotation shows what basic units of the chromosome are present. For example, FIGURE 4 shows a  $k\ l\ m\ g$  type chromosome, since all four structural units are markedly developed. On the other hand, FIGURE 2 shows  $k\ m\ g$  types, the

standard for later stages. In FIGURE 3, the frog chromosomes are  $k l m g$ , while the salamander pair are  $k m g$  types. The value of this system may be seen where large numbers of chromosome photographs need to be compared and where homologies between widely differing species of animals and plants are sought. Thus, the *Chironomus* salivary gland chromosome, described so well by Dr. Ethel Glancy D'Angelo (1946), would be of the  $k m g$  type. Tissue cell chromosomes in mitosis appear as  $k m$  types, since the granules

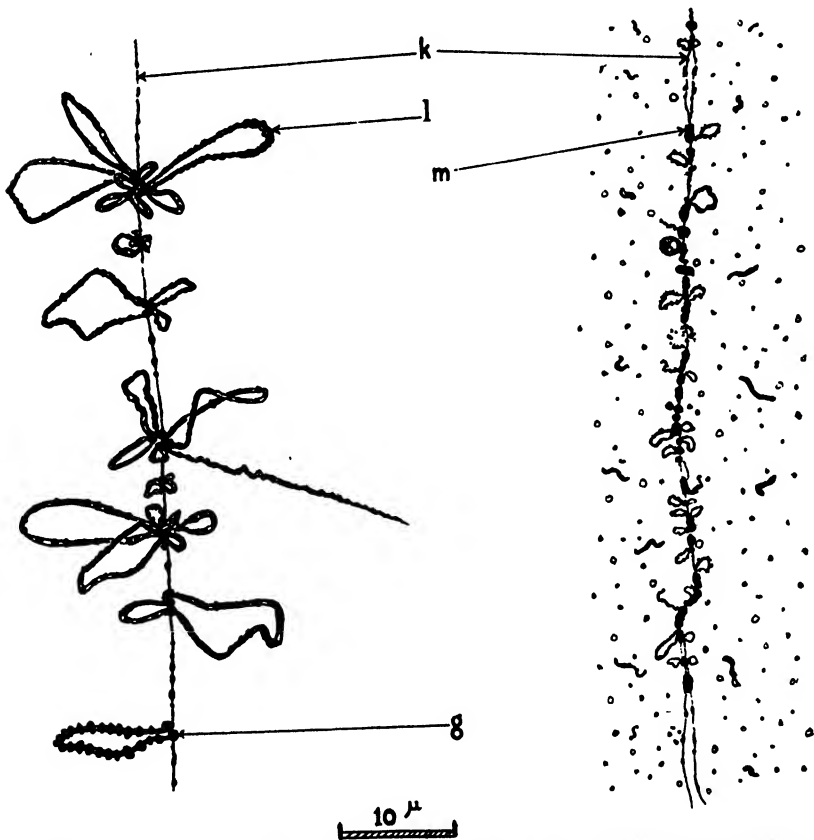


FIGURE 4. Diagram of normal amphibian egg chromosome structure at Stages 4 and 5. Stage 4 chromosome at left consists of lateral loop ( $l$ ) clusters of varying type and numbers held together by an apparently single chromonema ( $k$ ). Loops originate from paired granules ( $g$ ). Stage 5 chromosome at right shows many loop fragments and few residual broken and disintegrating loops. Matrix material ( $m$ ) coats the chromonema, which only rarely shows evidence of a split.

are not visible. Chromosomes in the male germ cells of amphibia, which normally pair with those described in this paper, have also been observed as  $k m$  types. But it should be emphasized that all egg chromosomes brought to their isoelectric point of pH 4.5 (Duryee 1941) or stained with basic dyes show there are granules present. Chromosome formulae may easily be determined from rapid smear preparations such as illustrated in PLATE 2 (A).

The following tabulation gives the formulae for the chromosomes in the accompanying photographs:

<i>Plate number</i>	<i>Chromosome formula</i>
2 (A)	$k\ m\ g$
(B)	$k\ l\ g$
(C)	$k\ l\ g$
3 (E)	$k\ l\ m\ g$
4 (A)	$k\ l\ g$
(B)	$k\ l\ g$
(C)	$k\ l\ m\ g$
5 (A)	$k\ m\ g$
(B)	$k\ g$
(C)	$k\ m\ g$
(D)	$k\ m\ g$
(E)	$k\ m\ g$
(F)	$k\ g$
6 (A)	$k\ l\ m\ g$
(B)	$k\ m\ g$

#### *Chromosomal Function*

The cytological evidence analyzed in this section leads to two general conclusions. One is that the germinal vesicle chromosomes control the synthesis of new material in the form of lateral loops, which grow outward and then dissolve into a host of barely visible particles—the loop fragments. The second is that nucleoli of larger size are produced at definite loci and, likewise, accumulate in the nucleoplasm. Both of these ideas have long been current. Indeed, Rückert, in his classic paper, clearly stated that the side loops of *Pristiurus* chromosomes are converted into nuclear granules. However, no experimental analysis of this important problem in germ cells has been made. The present discussion will be limited to physical studies on chromosomes in known chemical media. For more strictly chemical data on amphibian germinal vesicles, the reader is referred to the paper by Gersch, who used my isolation techniques and media.

#### *Loop Production and Variations*

Lateral loops begin to grow outward in Stage 3 nuclei and reach their maximum in Stage 4. The base of each loop always appeared associated with definite granules in the  $k$  thread, which stained more intensely than loop material. Attention is directed to PLATE 2 (B and C). The originating granules within each of the small clusters are clearly shown. There is no trace of loop material in the spaces between clusters. By the simple expedient of removing the nuclear membrane and adding a drop of 0.1 M.  $\text{NaH}_2\text{PO}_4$ , especially clear observation was possible. No case of "coiling" from one cluster to the next has ever been noted.

Loops varied greatly in size, not only between different clusters but also in the same cluster. Figures reported here are measurements made on chromosomes at the isoelectric point, while the ocular micrometer scale was

read critically to one micron plus or minus 0.5 at a magnification of 1600 X. On freshly isolated material, the measurements are correspondingly from 5 to 20 per cent larger, but were more subject to error on account of lack of definition. In frog material, Stage 4 chromosomes in a single nucleus had loops projecting 2.5 to 24  $\mu$  from the *k* thread. Average lateral projection was 9.5  $\mu$ . Urodele structures were relatively much larger. For example, the average lateral extension of loops in *Triturus pyrrhogaster* was close to 15  $\mu$  with extremes of 2 to 36  $\mu$ . PLATE 2 (B) shows a typical mixture of large and small loops in a single cluster. The photograph also brings out the fact that the number of loops per cluster varies from one to nine. Adjacent clusters on the same chromosome usually have different numbers of loops. This is also apparent in PLATE 4 (A).

The structure of each individual loop is of great importance. FIGURE 4 and PLATES 2 (B) and 4 (B) show that the loop consists of a hyaline cylinder approximately one micron in diameter. Embedded in this cylinder are the denser particles averaging 1.5  $\mu$  whose fate is discussed in a following section on "Loop Transformation." They are clearly not optical sections of "minor coils." When freed from the chromosome frame gel, as shown in PLATE 4 (A and B), the cylinder will contract into wavy lines, but the particles are still observable *in situ* and do not correspond with the irregular angles thus formed. Addition of standard fixatives or coagulating reagents produced and accentuated such artifacts, but the embedded particles remained discrete bodies.

Not all the loops on a single chromosome were alike. In *Triturus pyrrhogaster*, there always appeared one pair of chromosomes, each having a single unusual lateral loop. This was a heavily beaded structure, each bead being about 7  $\mu$  in diameter. At the corresponding loci, each loop originated from a pair of granules in the *k* thread, as indicated in FIGURE 4. The fact that loops can be different indicates that they are not parts of a continuous thread.

*Loop Numbers.* While it has been shown that the loop numbers vary from one unit cluster to the next and are not necessarily multiples of one another, the number of clusters per 100  $\mu$  of any chromosome in a single nucleus is remarkably constant. Some sample calculations based on the accompanying photographs will make this clear. For the purpose of comparison, a standard length of 100  $\mu$  has been selected. The chromosome in PLATE 2 (B) has approximately 23 loop clusters or centers of activity per 100  $\mu$ . Projecting from these loci are 71 loops  $\pm$  8. Allowing an additional 15 per cent for loops which do not appear, being above or below the focal plane in the photograph, it can be estimated that there are about 80 to 90 loops per 100  $\mu$  unit of length. The ratio of 80:23 shows that at Stage 4 there is a rough average of about 3.5 loops per cluster.

In more advanced stages, the average drops. This is brought out in PLATE 3 (E), which is a photograph of Stage 5 *Triturus* chromosomes. The long pair with three chiasmata measures 435  $\mu$ . Approximately 120 chromomeres or centers of activity can be counted in each chromosome. Therefore, there is an average of 28 chromomeres per unit of 100 microns, which

in comparison with Stage 4 indicates a slight shortening of the *k*-thread. On these same chromosomes, approximately  $213 \text{ loops} \pm 14$  can be counted. This gives an average of about 49 loops per 100 micron unit. The ratio of 49:28 gives an average of 1.7 loops per chromomere of cluster at Stage 5, in comparison to 3.5 at the earlier stage. By Stage 6, only a few loops could be found on the chromosomes. Subsequently, the averages become still lower, approaching zero. In summary, the number of loops per chromomere decreases with time, although the total number of chromomeres remains constant.

**Loop Solubility.** Lateral loops were dissolved off the chromosomes in a variety of ways without affecting the *k* thread. Solvents included dilute hydroxides (0.1 N) and bicarbonates and basic phosphates, the more hydrating anions of the Hofmeister series—such as Sulphide, Iodide, Thiocyanate and Citrate, and also KCl (0.01 M). Irradiation with X rays (over 50,000 r) and ultraviolet containing a strong band at 2537 Å also caused dissolution of the lateral loops.\* The X-ray experiments have been done with many hundreds of isolated nuclei, both frog and urodele, in the middle stages. All gave the same result, namely, that loops so treated were transformed into granules. On reacidification to pH 4.5, the chromosome pairs reappeared exceptionally sharp and with the same number of chiasmata, but without lateral branches. No evidence of any resorption back into the *k*-thread has been found. PLATE 5 presents six photographs of different types of chromosomes from which the lateral loops have been removed by various means. Whether or not matrix material remained after treatment depended upon the type of ion used and on the duration of exposure.

Reagents applied directly to isolated chromosomes gave clear results. In favorable experiments using very thin films (where reagent penetration was slowed by pressing down the cover slip into the plastic cover slip supports), actual disintegration of the loops was observed directly. Particular attention was paid to the chromonemata. No breakage or significant change was observed in them. In PLATE 5 (B), a pair of *Triturus pyrrhogaster* chromosomes is shown in which the chromonemata are twisted at the chiasma. All remaining lateral loops were dissolved off by short exposure to 0.01 M KCl. PLATE 5 (C) shows a single *Triturus* chromosome after momentary treatment with 0.1 M  $\text{Na}_3\text{PO}_4$  followed by 0.003 N HCl. Although the terminal nucleolus was preserved, the lateral loops went into solution and were converted into granular fragments. PLATE 5 (D) illustrates a "Q" chromosome from a Stage 6 *Triturus* egg. Loops have normally been dissolved away at this stage.

Extremely heavy dosages of X rays removed the loops completely from chromosomes even after they had been isolated. PLATE 5 (E) shows the effect of 100,000 r on an isolated pair in a thin film of Ca-free Ringer. It is evident that, while the *k*-thread has not been ruptured, all loops have disappeared. Dosages of 20,000 r fragmented few loops, in contrast to 50,000

\* The author wishes to express thanks to Dr. C. C. Clark of New York University for loan of a convenient U. V. irradiation device. This consisted of a quartz, mercury vapor discharge tube (1 cm. diam.) bent in the form of circle. It was mounted so that the microscope objective fitted through the circle. Isolated nuclei were irradiated through quartz cover slips 1 cm. from the source for 300 seconds. Sixty seconds of irradiation produced no effect.

r, which caused complete dissolution and left the chromosomes a granular hyaline cylinder. PLATE 5 (E) also demonstrates a high degree of correspondence between the similarly situated *g* granules in each chromosome. X rays did not remove the matrix; consequently each one is seen to be of the *k m g* category.

The complete transformation of a *k l m g* type chromosome into a *k g* form is illustrated in PLATE 5 (F). Here, 0.1 M  $\text{Na}_3\text{PO}_4$  was allowed to act for two minutes before acidification to pH 4.5. Only a central thread and some embedded granules remained, although the chiasma was still clear. The granules appeared as lumpy discrete masses. Since both Painter (1942) and Brachet (1929) have described these granules as Feulgen positive and since all their physical properties resemble the dark bands in insect salivary chromosomes, it seems possible that they are rich in desoxyribose nucleic acid. On the other hand, my own Feulgen preparations, under ordinary optics, have consistently shown all portions of Stage 4 chromosomes as negative; yet loops, granules, and chromonemata were clearly visible on switching to phase-contrast. Attention is drawn to the facts that in a chromosome pair not only is the spatial distribution of the granules along each *k*-thread a mirror image of the other, but, also, the size and shape of each granule corresponds in both homologues. As in the previous cases, no evidence of loop retraction was observed. It is concluded from the experiments typified by PLATE 5 (F) that matrix material can be completely removed leaving only the *k g* fiber.

*Micromanipulation Experiments.* The micromanipulative technique is well suited for many kinds of tests on chromosome function. Dissection of component parts revealed differences in physical properties. Differential solubility of loops and nucleoli were further examined by micropipetting reagents directly on the parts concerned. Chromosomes were operated with a Chambers instrument. After the nuclear membrane had been removed, the material was suspended in a hanging drop of calcium-free Ringer. PLATES 3 and 4 illustrate various types of experiments carried out.

In order to test whether the lateral loops were continuous with the longitudinal *k*-thread, a series of chromosomes were stretched in different salt media. In every case, both with anurans and with urodeles, individual loops or clusters of loops became separated from one another as units, *without the bases of the loops opening up*. No evidence of breaking of a hypothetical coil was seen even under oil immersion. Adjacent loop clusters could be separated over  $30\ \mu$  until the *k*-thread could no longer be seen. On approximating the needles, *k*-threads reappeared as before and the chromosomes resumed their original appearance, matching their homologues exactly. Each stretched chromonema was found to be a single thread with slight oval bulges all of much smaller size than that of the loops. The original photograph in PLATE 4 (A) shows this condition plainly.

When matrix (*m*) substance was present, it separated on stretching into unequal segments as shown in PLATE 4 (A). It separated easily as a viscous material with no trace of snapping or breaking fibrillae, such as Ris has postulated. On approximating the microneedles, matrix segments fused or



molded together restoring the original appearance. This type of experiment is shown in PLATE 4 (C). As reported in an earlier paper (Duryee 1941), the relative elasticity of the *k*-thread varied from an average increase of +450 per cent in calcium-free media (pH 6.8) to only +85 per cent in the same medium acidified to pH 4.2. In 0.001 M  $\text{CaCl}_2$ , a chromosome could be stretched only about twice its length before breaking. Recent experiments have confirmed the fact that, in contrast to *k*-thread elasticity, the loop material (*l*) is always more brittle, less elastic, and more frangible. In no case could a loop segment be stretched more than 50 per cent before breaking. Such an experiment is shown in PLATE 4 (B). The conclusion is that the physical properties of loop material are markedly different from those of the *k*-thread.

*Loop Transformations.* It has been brought out by the foregoing sections that lateral loops originate from chromosomal granules and that the lateral branches themselves are not homogeneous in structure, but are made up of smaller particles embedded in a hyaline cylinder which has different solubility properties from the *k*-thread. This section deals with the transformations of the component particles of loop material into many separate nuclear granules or loop fragments. Reference is made to FIGURE 4. As mentioned previously, under favorable conditions with hydrating reagents added slowly and in minimal quantities, actual dissolution of the loops with liberation of embedded granules could be followed under oil immersion. The hyaline bodies so formed were identical in size, appearance, and position with those normally formed in a cell. Stage 4 chromosomes of *Triturus* given 30,000 r of X-irradiation always showed many lateral loops missing. In such places, many loop fragments were found adjacent or attached to the chromosomes. It is strongly suggested by these experiments that the characteristic normal loop fragments are produced in a similar fashion to the experimental ones.

One of the most constant normal features of freshly isolated Stages 5 and 6 nuclei was the cloud of hyaline refractive bodies seen surrounding and emerging from the central chromosome area. They are illustrated in the photographs of PLATE 6. In diameter, they measured 0.5 to 2.0  $\mu$ . Staining with basic dyes, solubility and position in the nucleus classes them with the larger nucleoli. On the other hand, constant size, refractive surface, and spherical shape differentiate them from ground-substance floccules. Loop fragments do not begin to appear until late Stage 4, when chromosome loops are beginning to disappear. They are numerous in Stage 5 and increase to between 70,000 to 100,000 by late Stage 6. It is highly significant that, in those earlier stages where lateral loops are most developed, the loop fragments are lacking. All the observations support the inference that loop fragments are accumulated products of lateral loop breakdown.

It is of interest in this connection that animals which had been long in captivity or on low nutritional level usually had degenerating eggs in which lateral loops have disappeared. In such eggs, loop fragments were numerous. These observations show that nuclei possess autolyzing properties

and, therefore, that attention must be paid to the feeding of the laboratory animals. The observations also show that a differential susceptibility exists between the *k*-fiber and the lateral loops. It is concluded that loop transformations in degenerating eggs are merely accentuations of normal processes that may be observed in every normal egg of the proper stage.

### *Nucleolar Production*

As has been pointed out, the physiology of germinal vesicle chromosomes consists of two types of intricate mechanisms. The cytological evidence for the first of these, namely, lateral loop production and transformation of loops into loop fragments, has been presented in the foregoing sections. The second type, namely, nucleolar production, is discussed here.

Amphibian nucleoli are relatively large intranuclear inclusions from 6 to 25  $\mu$  in diameter. They possess multiple vacuoles that are capable of various transformations, such as fusion, eversion, and phase reversal. Under abnormal conditions, nucleoli may be made to fuse into irregular masses over 140  $\mu$  in diameter. Their general features and normal distribution are schematized in FIGURE 1. Microphotographs of them appear in all the plates, but particular attention is called to PLATES 1, 3, and 5. PLATE 1 (A and B) shows that in earlier stages they are limited to a peripheral position. Later, they take up a more central position in Stage 5 and 6 nuclei [cf. PLATE 1 (E)]. When the germinal vesicle breaks down in normal eggs at the time of ovulation, all the nucleoli dissolve slowly and their substance then becomes part of the cytoplasm. It would be interesting to know what role is subsequently played by these proteins. PLATE 3 (A) shows nucleoli that have migrated out into the sacs on the nuclear membrane which protrude into the cytoplasm.

All observations agreed in showing that nucleoli arise at specific loci on the chromosomes. From the point of origin they migrated to the periphery, as shown in PLATE 1 (B). As later waves of production took place between Stages 3 and 4, their numbers increased, as shown in PLATE 1 (C). Up through Stage 3, newly formed, central nucleoli were attached directly to specific chromosomes by lateral branches. Centrifugal migration appeared to be aided by expansion of the chromosome frame. It was possible to reverse the process partially by contracting the frame with dilute solutions of heavy metals, acids, and basic dyes. In cases where the nucleoli had already reached the periphery and had become adherent on the inside of the nuclear membrane, shrinkage of the frame with the major parts of the chromosomes occasionally placed severe tensions on the nucleoli via the lateral branches. Such stretched nucleoli were pear-shaped or had two cones on opposite poles. PLATE 3 (B) shows similar distortion produced by microneedles. The elasticity of the nucleolus under nearly normal conditions was readily demonstrable. When later stage nucleoli were found attached directly to the *k*-thread, as illustrated in the photograph of PLATE 5 (C), it was not possible to dissect them free without fracturing the chromosome. No by-passing of nucleoli by the *k*-thread was ever observed. It

was concluded that Stage 5 nucleoli are produced, not on branches as earlier ones were, but actually embedded in matrix substance and therefore firmly attached to the *k*-thread.

The problem of the structure and chemistry of nucleoli is very complex. The data on salt effects (*cf.* Duryee 1941) were found to conform with the principle that nucleoli are colloidal coacervates as defined by Bungenberg de Jong and Otto Bank.\* It must be kept in mind that their classical observations on the morphological phenomena occurring in the case of coacervation of biocolloids are highly pertinent to the problem of nucleolar structure. For example, the typical solubility of nucleoli in dilute alkalis or in any of the common buffers can be blocked at progressive stages by appropriate neutralization. Experiments on many thousands of nucleoli have proved that nucleolar solubility is a colloidal coacervate phenomenon that takes place in stages. First, the outer shell disintegrates, freeing one or more interior droplets. Next, the inner droplets may dissolve, coalesce, or form chains, depending on the time interval between addition of the peptizing reagent and the flocculating one. The following simple rules were found that brought about internal phase reversals, eversion of contents to produce *amphinucleoli*, fragmentation, fusion, and dissolution of nucleoli.

*Dissolving.* Reagents like distilled water, hydroxides of Na and K (0.01 M), KCl 0.1 M, basic phosphates, bicarbonates, and the hydrating anions of the Hofmeister series (*e.g.* sulphide) all caused the nucleolar membrane to dissolve, followed by dissolution of the polyphasic interior. In all of these cases, complete disappearance of nucleoli was observed. Especially interesting was the effect of 0.1 M KCl, in which nucleoli were dissolved, but the chromosomes remained suspended in the central chromosome frame.

*Fragmentation.* Fragmentation of nucleoli is defined as breakdown of the nucleolar membrane with the escape or release of contained vacuoles or granules. Criteria of fragmentation could be precisely determined with an ordinary 4 mm. high-power objective. The resultant picture was a small clump of granules or vacuoles, slightly larger in diameter than the pre-existing nucleolus. Substantially, all of these reagents listed, which cause nucleolar dissolution, also produced fragmentation when added cautiously in amounts of the order of 1 mm.<sup>3</sup> per 4 mm.<sup>3</sup> of N-medium. Fragmentation instead of dissolution was also favored by slowing the rate of addition of the hydrating reagents and by arresting or neutralizing the action of the agent. NaCl 0.02 M produced smaller nucleolar droplets than did NaCl 0.05 M. Fragmentation and eventual solvation was also produced by hypertonic concentrations of the order of 0.5 M, whereas the nucleoli were always insoluble in 0.1 M NaCl. It must be concluded from these experiments that nucleoli are colloidal in character, behaving as viscous emulsoids, best described by the term "coacervates." It further follows that, since Bouin's and dilute formaldehyde also cause nucleolar fragmentation and eversion of contents, the term "amphinucleoli," used by the older cytologists, applies to a colloidal artifact.

\* Bungenberg de Jong and O. Bank defined complex coacervation as "the mutual flocculation of two oppositely charged biocolloids, in which the flocculated substance has the nature of a liquid." Non-living models are formed from mixtures of negatively charged colloids such as clupeine, gelatin, serum albumin, or egg albumin, with positively charged colloids like gum arabic or Na-nucleinate.—*Proc. Kong. Nederlandsche Akad. V. Wetenschappen*, Vol. XLII, 1939.

*Fusion.* The coalescence of nucleoli was frequently observed. Several examples of fused nucleoli are shown in PLATE 1 (F). The conditions that bring about fusion were found to involve two basic factors. One is a partial or complete solvation of nucleoplasmic gel to allow the physical displacement of nucleoli. The other is surface change of the nucleolus itself that permits coalescence. These conditions are satisfied by a variety of the hydrating anions, but most efficiently by application of high dosages (60,000 r) of X rays or by NaCl solutions of the order of 0.5 M. The fact that nucleoli can coalesce is further proof of their emulsoid nature.

*Eversion through the Nuclear Membrane.* Experiments with dilute acids, especially  $\text{H}_3\text{BO}_3$  0.1 M, showed that peripheral nucleoli, in contrast to the larger central ones, could be made to evert their contents through the nuclear membrane. An examination of transparent eggs (Stage 3) treated with the reagent usually showed delicate translucent hemispheres projecting 10 to 30  $\mu$  into the cytoplasm. There was always a direct one-to-one correspondence between each surface hemispherical "bubble" and each flattened nucleolar remnant on the inside of the nuclear membrane. Observations of the surface of the nuclear membrane in all the later stages revealed multiple rounded structures in the membrane substance which correspond to nucleolar remnants. These seem to warrant the conclusion that the process of nucleolar eversion through the membrane is also a mechanism by which the nuclear surface area is increased.

### Discussion

The data presented in this paper, when taken together, form an integrated picture of how a chromosome functions in a cell. Lateral loop chromosomes are characteristic of the large eggs of vertebrates, notably in teleosts, amphibia, and aves. Since cleavage patterns of the egg represent primarily a distribution of materials already formed during a protracted growth period in the ovary, it is only natural to expect that the egg nucleus should possess a special mechanism for synthesizing the needed substances for later development. This is particularly important in forms, like the amphibia and fish, where the nurse cell mechanisms are not as fully developed as in the higher vertebrates. It has been brought out by this new cytological technique that there are two major methods for accumulating chromosomal products in the germinal vesicle. One method is the production from lateral loops of a large number of loop fragments, estimated to be 70,000 to 100,000. The other is by successive waves of nucleolar production, some of which result in direct contribution to the cytoplasm through the nuclear membrane, and some by belated admixture at the time of germinal vesicle breakdown. The colloidal coacervate characteristics of these nucleoli support the hypothesis that they are interaction products of oppositely charged colloids, such as nucleic acids with basic proteins. Whatever the mechanism may be, it is clear that nucleoli are produced at a limited number of loci on the chromosomes.

While the basic facts of nucleolar production described in detail by many authors are in general agreement with those reported here, there is an alternative hypothesis to explain the normal disappearance of lateral loops during

ovogenesis. This alternative can be called "retraction theory" sponsored by Ris (1945) and by Painter (1940 and 1942). It assumes, essentially, that the lateral loops are "merely gyres of the major coil of the chromonema" and that the chromosomes of the egg are not diplotene but "polytene" corresponding to a reduplicated condition proposed for the inset salivary gland chromosome. It supposes that lateral loops do not dissolve off, but are retracted back into the chromosome. Since there are at least six basic experimental and analytical reasons why the *lateral synthesis* theory developed in this paper fits the known facts better than the *retraction theory*, they are reviewed in detail.

In the first place, actual fragmentation of lateral loops into discrete filaments and granules without rupture of the chromonemata or change in length of the chromosomes has been repeatedly observed. Among the great variety of hydrating reagents that produces this result, ordinary amphibian River solution buffered with  $K_2HPO_4$  stands out as a physiological example. Recent experiments with X-irradiation of *Triturus pyrrhogaster* eggs at the 20,000 to 60,000 r levels gave all stages of loop fragmentation without chromonemata breaks. No case of loop retraction with any reagent has been observed. The facts of solubility and the actual observation of loop disintegration clearly disprove any possibility of retraction.

Secondly, the experiments of stretching chromosomes with microneedles did not open the lateral loops. On the contrary, multiple loop clusters were separated and could be seen to be held together by a single thread. It is recognized that the *k*-thread may have a latent cleavage plane which would correspond with tetrad formation, but indications of duplication were found only in rare instances. Conversely, it was possible to cut and tear many loops along a small segment of chromosome without affecting the stretching properties of the *k*-thread.

Third is the fact of normal decrease in number of loops with advancing oogenesis. By late Stage 6, the average number of lateral loops per chromosome diminished toward zero, and the longest chromosome measures only 40  $\mu$  and several pair are less than 10  $\mu$ . The average thickness of each chromosome cylinder is approximately 1  $\mu$ . If the retraction theory were valid, it would be necessary to explain how the previously expanded chromosomes and loops could be compressed into such a small volume. The following example may serve to illustrate this point. A Stage 4 *Triturus* chromosome has lateral loops that project laterally an average distance of at least 15  $\mu$ . Since the loop is double, the average loop filament length is 30  $\mu$ . From the photograph in PLATE 2 (B), it was estimated that there are at least 80 loops per 100 micra of chromosome length, and the longest pair at its iso-electric point is at least 700  $\mu$ . Multiplying  $30 \times 80 \times 7$  gives a total loop filament length for one chromosome at 16,800  $\mu$  or 16.8 mm. It would be impossible, obviously, for this figure to shrink to 40  $\mu$ , the length of the largest chromosome ready to go on the 1st maturation division spindle, without loss of material or extraordinary thickening. On the other hand, with the mechanism of lateral loop sloughing, as put forth in this paper, no such difficulty arises. The *k*-thread never exceeds 750  $\mu$  and it is then so

thin as to be almost unobservable under oil immersion. If such a Stage 4 chromonema is shrunk experimentally with  $\text{CaCl}_2$  down to  $100\ \mu$ , it does not thicken to more than  $1.5\ \mu$ . On this basis alone, the retraction theory is clearly untenable. In this connection, it is of interest that Dodson (1949) has calculated the specific surface of *Amphiuma* oocyte chromosomes as 108.37 square meters per gram of tissue.

In the fourth place is the fact of variability in loop numbers per cluster. Previously, it was shown that the average number of loops per cluster diminished from 3.5 at Stage 4 to 1.7 at Stage 5, and later approached zero. It was also shown that different numbers of loops exist in the various clusters on the same chromosome. It is significant that these distribution differences along the chromosome were not noticed in the old methods of fixing, sectioning, and staining. If egg chromosomes are assumed to be diplotene, as most classical cytologists long ago agreed, then two and only two loops per cluster should appear. The fact that the loops vary from one to nine along any chromosome strongly suggests synthesis going on at different rates, rather than "gyres of the major coil." This conclusion is further borne out by the fact that lateral loops within any one cluster are usually of different sizes. When amphibian genetics and microchemical methods are more advanced, it might be expected that successive "crops" of loops might be observed and quantitatively measured.

Fifth is the problem of variation in the finer details of individual loop structure. From the standpoint of the lateral synthesis theory, granules embedded in a single loop filament represent substances produced at a specific chromosome locus and are presumably qualitatively alike. Similarly, if one assumes that there is a specific locus responsible for production of each of the complex chemicals required by the embryo—such as thyroxine, haemoglobin, and acetylcholine—then lateral loops in different clusters should contain qualitative differences. Unfortunately, microchemical methods are not yet sufficiently delicate to detect such differences. However, there is at least one, and possibly two more, morphological differences detectable between lateral loops. In *Triturus pyrrhogaster*, as has been pointed out, there is one pair of chromosomes each having a single, heavily beaded lateral loop at homologous positions. The fact that these two are markedly different from their neighbors does not fall in with the coil theory. Indeed, the point that each is a *single* structure suggests further that no question of "polyteny" is involved. The simpler explanation suffices, *i.e.*, that the heavy loop is a synthetic product of a specialized locus.

In the sixth place are the physical differences between the lateral loops and the longitudinal *k*-thread. The former are measurably more brittle, frangible, and less elastic than the chromonemata. When stretched with microneedles, any small segment of the *k*-thread could be increased from 140 to 900 per cent of its length without breaking. Lateral loop filament segments could never be stretched over 50 per cent without rupture. These facts taken together with the differential solubility data contradict the assumptions of Ris and Painter that lateral loops are portions of the chromonemata,

Finally, the *lateral synthesis* theory is adequate to explain the facts of nucleolar accumulation, production of loop fragments, and growth of the nucleus at the same time it allows for contraction of the chromosomes preparatory to going on the 1st maturation spindle. Photographic evidence has been offered showing that nucleoli originate at definite loci attached to the *k*-thread, but it is not yet known whether the matrix substance also coats the early nucleoli. It is certain, however, that nucleoli continue to increase in numbers, not haphazardly in the nucleoplasm, but in the definite area at the center of the nucleus between and immediately surrounding the chromosomes. This is also the area where loop fragments appear as the crops of loops disappear. The adoption of a chromosome formula (combinations of the letters *k*, *l*, *m*, *g*) focuses attention on the four basic units of chromosome structure: the chromonema (*k*); the self-duplicating granule or gene, as some may wish to call it (*g*); the lateral loop observed to consist of separable particles embedded in a hyaline fibril (*l*); and the chromosomal matrix (*m*) or coating material, which may also possibly contribute to the loop. The chromosome formula has already been useful in orienting microchemical experiments on nuclear components, while the retraction theory is hardly more than a sterile hypothesis based on unreliable methods.

It is relevant to this discussion that lateral chromomere loops are in some way dependent on the nutritional level of the animal as well as on the physiological state of the egg. It has often been observed that in animals which have not fed for long periods of time many of their ovarian eggs are being resorbed. Isolated nuclei from such ovaries usually exhibit ragged or fewer loops per chromosome. On the other hand, the best developed loops are seen in the appropriate stages of eggs from the healthiest individuals. Clearly, the dependence of loop structure on nutrition level supports the concept of lateral synthesis.

The experiments presented in this paper emphasize the importance of the nuclear polyphasic colloids in protecting and maintaining the chromosomes in conditions which favor lateral loop synthesis and nucleolar production. It is clear that chromosomes and nuclear substrate are highly interdependent. We are, thus, led to a general working hypothesis that the egg chromosome is a linearly expandable device to allow access of substrate to anchored template molecules or to key enzymes and thereby facilitate lateral synthesis. This view is in accord with some very recent observations on chromosome structure made by Buchholz (1947) using the electron microscope. His conclusion is that elongated longitudinal threads connect paired granules or chromomeres at the rate of  $4\text{--}5/\mu$ . From a consideration of eversion of nucleolar contents through the nuclear membrane and the widely recognized fact of normal germinal vesicle breakdown, it is apparent how chromosomal products are added to the egg cytoplasm at a very critical time preparatory to fertilization. These mechanisms might also account for accumulation in the cytoplasm of such postulated units as "cytogenes." While this paper has been confined largely to a morphological study, it has shown the fundamental dependence of nuclear morphology on the chemical constitution of the environment. Future experiments in chromosome physiology should

combine *both* colloid substrate chemistry and the experimental analysis of the units of chromosome structure. This in turn should lead to the now approachable, but still distant, goal of culturing chromosomes and their component parts outside of the cell *in vitro*.

### Summary

(1) Experimental analysis of over 1600 individually isolated nuclei of amphibian ovarian eggs has shown that the chromosomes have two basic mechanisms for producing material which is later transmitted to the cytoplasm. One is the production of many thousand filaments by a process of *lateral synthesis* from multiple loci along the chromonemata. The other is by production of many hundreds of nucleoli.

(2) Techniques of isolating and washing individual nuclei and nuclear components in physiological media, followed by minimal amounts of mild reagents, have been found to yield cytological detail superior to any method of fixing and staining. Methods included microdissection, pH changes with dilute buffers, and experiments with ultraviolet light and high intensity X rays. Species used include: *Rana temporaria*, *Rana pipiens*, *Rana calesbiana*, and *Triturus pyrrhogaster* and *Triturus viridescens*.

(3) Amphibian ovocyte development is characterized by six different stages or phases of nuclear growth. A diagram of the normal series for the frog is given.

(4) The 13 pairs of anuran germinal vesicle chromosomes have constant relative size and fixed numbers of chiasmata. Each pair therefore may be recognized and identified. They are designated: Q<sub>1</sub>, Q<sub>2</sub>, Q<sub>3</sub>, Q<sub>4</sub>, R<sub>2</sub>, R<sub>3a</sub>, R<sub>3b</sub>, R<sub>4</sub>, S<sub>4</sub>, S<sub>6</sub>, S<sub>7</sub>, T<sub>1</sub>, and T<sub>2</sub> respectively.

(5) Experiments show the chromosomes consist of 4 basic parts: the longitudinal chromonema or *k*-thread (*k*), the lateral loops (*l*), which originate from chromonema anchored granules (*g*) and a covering matrix (*m*). Formulae for variations from the basic type (*k l m g*) are: *k m g*, *k m*, and *k g*.

(a) Lateral loops occur in separable clusters of 1 to 9 loops along a single chromonema. There is no "coiling" from one chromomere cluster to the next. Each consists of a hyaline filament with embedded granules.

(b) Lateral loops reach their greatest development in Stage 4 when the chromosome frame is most expanded. In frogs, they may extend laterally 24  $\mu$  with an average of 9.5  $\mu$ . In urodeles, comparable lateral loops average 15  $\mu$  with extremes of 2 to 36  $\mu$ .

(c) Lateral loops both normally and experimentally fragment into hyaline granules approximately 1.5  $\mu$  in diameter. Loops are not resorbed back into the chromosome. At Stage 4, loop clusters average 23 per 100  $\mu$  of chromosome length with an average of 3.5 loops per cluster. By Stage 5, there are only 1.7 loops per cluster, and, at Stage 6, the number approaches zero. The number of loops per chromosome decreases with time, although the total number of chromomeres per chromosome remains constant.

(6) Nucleoli arise at specific loci on the chromosomes. They are colloidal



in character, behaving as viscous emulsoids, best described by the term "coacervates."

- (a) Nucleoli dissolve in distilled water, dilute hydroxides, and KCl 0.1 M. They are insoluble in NaCl 0.1 M.
- (b) Fragmentation, with release of internal granules or vacuoles, is produced by a wide variety of hydrating anions and fixatives.
- (c) Fusion of nucleoli can be caused by the action of X rays or hypertonic NaCl.
- (d) Nucleoli evert their contents through the nuclear membrane, when transparent cells are treated with dilute acids. This is also a process whereby the nuclear membrane area is increased.
- (7) The wide significance of this chromosome *lateral synthesis* theory in connection with the principles of germinal vesicle breakdown and preparation for fertilization is discussed. Also reviewed are the reasons for rejecting both the *loop retraction* theory and the concept of polytene strands on egg chromosomes.

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### *Discussion of the Paper*

DR. HANS RIS (*Johns Hopkins University, Baltimore, Md.*): One must agree with Dr. Duryee that the amphibian egg is a most interesting system and that its giant chromosomes are fascinating structures indeed. They seem to be ideal objects for the study of chromosome metabolism and nuclear-cytoplasmic interaction. Therefore, they have interested a great number of biologists since their discovery by Rückert.

The remarkable activities of the chromosomes during the growth of the egg, which Dr. Duryee just has described, are, of course, of great general interest, if they can be substantiated. However, if one has ever seen these chromosomes in the living germinal vesicle, faintly visible, tenuous strands and loops, one wonders how Dr. Duryee can be so confident in his views about their structure, based solely on observation of fresh material.

Now most cytologists will agree with Dr. Duryee that the study of living cells is of great importance and has too often been neglected; but they have also found that, for the study of the detailed structure of chromosomes, they could not rely on the observation of living material because the chromosomes are then usually very difficult to see. They have, therefore, worked out the techniques of fixing and staining chromosomes to make them more visible. I have looked at a great many different chromosomes in the fresh state and after staining and can only corroborate what Belar had already shown, namely, that fixation does not change the essential structure of chromosomes. Phase-contrast microscopy will, no doubt, extend the limits of living observation a great deal and perhaps make it possible to study chromosome structure in living cells. The cytologist will certainly want to use this wonderful new tool to confirm wherever possible in living cells what he found after fixation. But observations of living cells with the ordinary microscope alone are a very weak foundation on which to base views on chromosome structure.

In a study of these giant chromosomes in amphibian eggs after fixation and staining, I found that these chromosomes are not so totally different from other diplotene chromosomes as Dr. Duryee thinks. Like other diplotene chromosomes, they consist of several chromonemata with major and minor spirals. The gyres of the major coil form the characteristic "loops." The "loops," then, are part of the chromonemata themselves and not new structures built up by the "chromioles." Compared with most other chromosomes, the chromonemata are, however, enormously elongated. This interpretation agrees with what we know of other chromosomes. In egg cells of different insects, for instance, we can find all intermediates from small diplotene chromosomes to large ones, resembling amphibian chromo-

somes. Amphibian chromosomes are merely extreme in size, but then the salamander egg is an extreme cell also!

The dissolution and micromanipulation experiments of Dr. Duryee do not necessarily contradict this view. "Loops" may shrink or collapse upon the central core of the chromosome under the influence of the agents used and thus disappear without being dissolved. The stretching of the chromosome may break some of the chromonemata. Their "loops" would remain unstretched and they would form the observed clumps along the unbroken stretched strand.

I do not think that we can say anything at present about the way these chromosomes grow or take part in the growth of the egg and shrink again to the small size of the maturation divisions. We first need a thorough investigation of the structural changes of these chromosomes during oogenesis, making use of all cytological techniques available.

DR. W. R. DURYEE: The evidence against Dr. Ris's speculation that "the gyres of the major coil form the characteristic loops" has been presented in this paper. The fact that egg cells of insects have all intermediates from small diplotene chromosomes to large ones resembling those of amphibia is in favor of the view that lateral loop chromosomes are a widely distributed mechanism for synthesis in germ cells. One can not agree, however, with the statement that the salamander egg is an extreme cell. Compared with eggs of fish, reptiles, and birds it is both smaller and less specialized.

It is not likely that loops could disappear completely and still not be dissolved. Dr. Ris does not say how, if his theory were correct, all the "multiple" chromonemata except one breaks in every chromosome and between every cluster when the chromosome is stretched. Nor does he explain the mechanism of loop retraction. No experiment on any chromosome either within or without the nucleus has shown any retraction. All the experimental evidence is for loop fragmentation.

Dr. Ris upholds the point of view that cytologists should "confirm wherever possible in living cells what has been found after fixation." This seems like a backward philosophy. One would think, on the contrary, that the obsolete techniques of fixation and staining, if used at all, should be only to confirm what is found in the fresh, unfixed material. The experimental point of view is needed especially in the field of cytology, which has lagged so far behind its sister sciences in biology.

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PLATE 1 (See opposite page).

(A) *Rana temporaria*. Portion of ovarian wall showing young transparent eggs (Stages 1 and 2) and larger opaque eggs (Stage 3). Arrow indicates isolated nucleus from another egg (also Stage 3) which has been floated into the field.

(B) *Rana pipiens*. Young Stage 3 egg with nucleus *in situ*. Peripheral nucleoli visible beneath nuclear membrane. Microneedle inserted into center of chromosome area.

(C) *Triturus pyrrhogaster*. Stage 4 nucleus isolated in N-medium. Nucleoli embedded in chromosome frame gel.

(D) *Rana temporaria*. Stage 6 nucleus treated 0.01 M KCl to dissolve all internal structures except the chromosome frame, which coagulated with 0.005 M CaCl<sub>2</sub> appears as small ball that has sunk to the inside of the nuclear membrane.

(E) *Rana pipiens*. Early Stage 6 nucleus isolated in N-medium. Sac-like organelles protrude from the nuclear membrane. Cloud of central nucleoli surrounds and obscures the chromosome frame.

(F) *Rana temporaria*. Isolated central nucleoli and chromosome frame from a Stage 6 nucleus, coagulated with CaCl<sub>2</sub> 0.005 M.

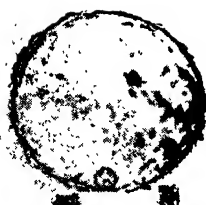


PLATE 1 (For description see facing page).

## PLATE 2 (See opposite page).

(A) *Triturus viridescens*. Smear preparation of late stage 5 nucleus, showing long chromonemata. Nearly all lateral loops have normally previously been sloughed off.

(B) *Triturus pyrrhogaster*. Oil-immersion micro-photograph of Stage 4 chromosomes *in situ*. One portion (c. 100 $\mu$ ) of a chromosome pair is in sharp focus at center. Note the individual loop clusters connected by a single longitudinal thread or chromonema. Details of individual loop structure are in sharp focus at various places throughout the picture.

(C) *Triturus pyrrhogaster*. Low power view (optical section) of isolated Stage 4 nucleus. Slightly stained with 1:8,000 crystal violet in N-medium. Note wide distribution of chromosome pairs with large lateral loops in expanded chromosome frame. Both peripheral and central nucleoli can be seen.

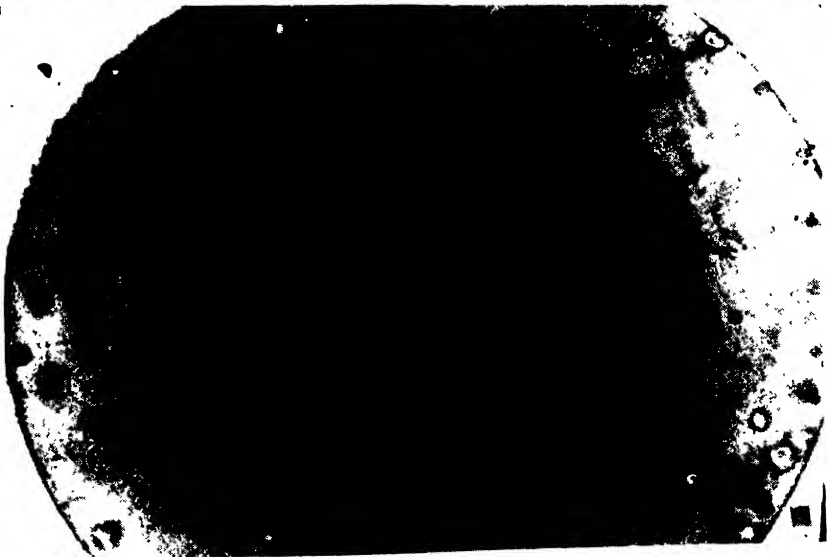
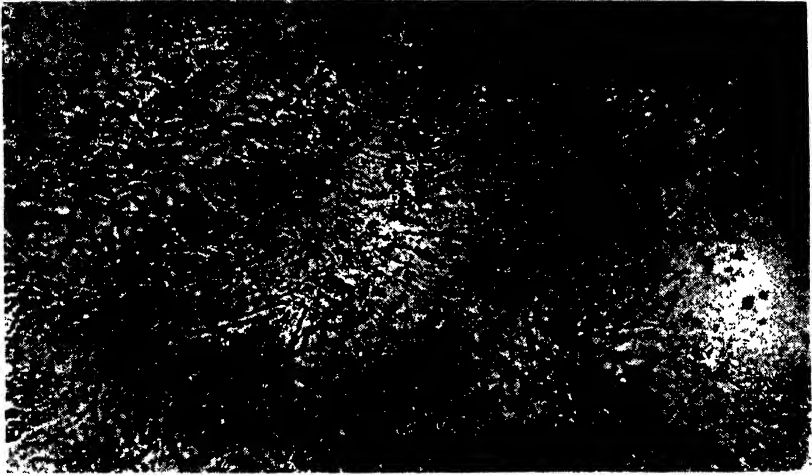
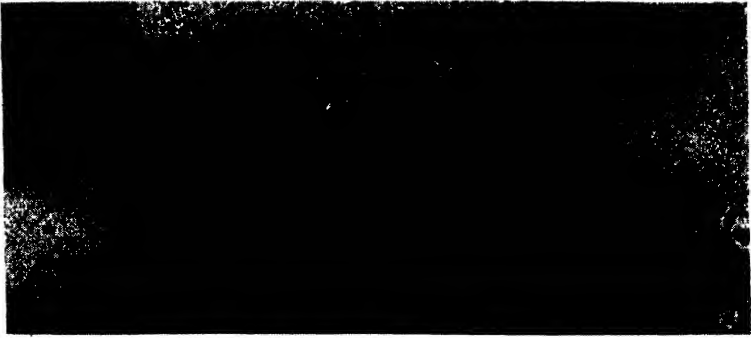


PLATE 2 (*For description see facing page*)

## PLATE 3 (See opposite page).

(A) *Rana pipiens*. High power view of equator of Stage 6 nuclear membrane showing details of membrane sacs. A nucleolus has escaped into one sac.

(B) *Triturus pyrrhogaster*. Nucleolus being stretched with microneedles.

(C) and (D) *Triturus pyrrhogaster*. Nucleolus before and after microdissection. Note torn edge of nucleolar substance in D showing that at Stage 5 the emulsoïd coacervate interior is a gel. The elasticity of the nucleolar capsule is also indicated.

(E) *Triturus pyrrhogaster*. Pair of Stage 5 chromosomes being stretched with microneedles. Note reduced numbers of lateral loops and their smaller relative size. Numerous nucleoli and loop fragments are visible in background



PLATE 3 (*For description see facing page*).



## PLATE 4 (See opposite page).

(A) *Triturus pyrrhogaster*. Stage 4 chromosome from same nucleus shown in Plate 2 (B). Note that stretching the chromonema does not cause the lateral loops to open. Different numbers of loops can be seen in adjacent clusters. The fine chromonema appears single and altogether different from the broken loop filament projecting at right angles on the right.

(B) *Triturus pyrrhogaster*. Micro dissection of a single lateral loop. The fine granular structure of individual loop filaments is in sharp focus at several places. Collapsed loops are matted on a small section of a chromosome now that the chromosome frame has changed to a sol. Note that the average diameter of the matted section is many times greater than that of the chromonema in (A) above. From same nucleus as (A).

(C) *Triturus pyrrhogaster*. Stage 5 chromosome being stretched with microneedles. Nearly all lateral loops have normally disappeared, but few remaining can be seen. Chromonema or k-thread is more elastic at some points than at others. If elastic limit is not exceeded, it will, on reapproximation of the needles, take on its original appearance matching the control or homologous member of the pair on the left.

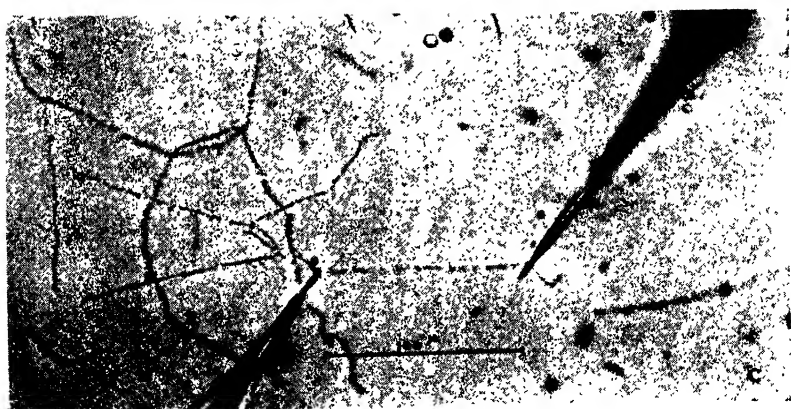
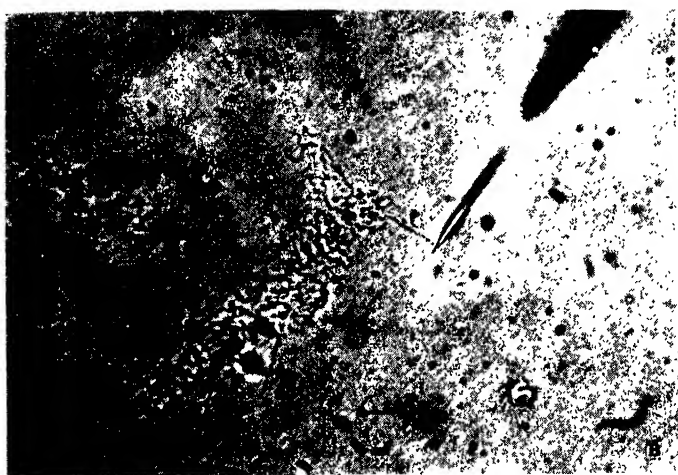


PLATE 4 (For description see facing page).

## PLATE 5 (See opposite page).

(A) Cutting a Stage 6 *Triturus* chromosome. The k-thread has been severed at two places in one chromosome; the homologue is still intact. Another chromosome pair is slightly out of focus at the right. Absence of loops and presence of matrix classes this as a *k m g* type.

(B) *Triturus pyrrhogaster*. Stage 5 chromosome pair previously treated with KCl 0.01M. Loss of matrix classes this as a *k g* type.

(C) *Triturus viridescens*. Stage 4 chromosome treated with  $\text{Na}_2\text{PO}_4$  0.1 M immediately followed by 0.003 N HCl. All lateral loops were dissolved and converted into granular fragments seen slightly out of focus in background. Note the large terminal nucleolus. (Oil-immersion.)

(D) *Triturus pyrrhogaster*. Ring or "Q" type chromosome pair from Stage 6 nucleus. Note swollen matrix, granules and the k-thread. Also the absence of loops. *k m g* type.

(E) *Triturus pyrrhogaster*. Stage 5 chromosome pair which had many lateral loops before receiving 100,000 r of X-rays. Note absence of lateral loops but persistence of the k-thread. Also the high degree of correspondence between granules in each chromosome.

(F) *Rana pipiens*. Transformation of a Stage 4 *k l m g* type into type *k g*. Isolated chromosome pair treated with  $\text{Na}_2\text{PO}_4$  0.1 M then acidified with HCl. Note loop fragments in background and correspondence of granules in the k-thread at right of chiasma.

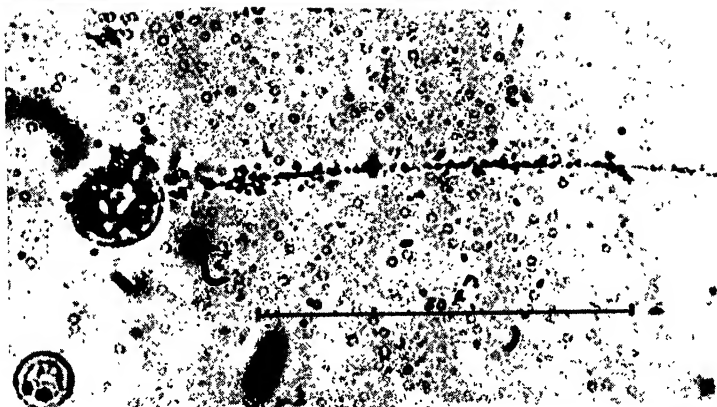


PLATE 5 (For description see facing page).

## PLATE 6 (See opposite page).

(A) *Rana catesbeiana*. Stage 5 nucleus isolated in N medium and brought to iso-electric point of pH 4.5. Chromosome pairs have only a few lateral loops at this stage. Accumulation of loop fragments adjacent to chromosomes is very marked. Note peripheral nucleoli in area outside of the chromosome frame.

(B) *Rana catesbeiana*. High power view of central area of Stage 5 nucleus. Large numbers of loop fragments are visible between the chromosomes, which are now of the *kmg* type. Nucleoli are shown immediately outside the central chromosome frame.

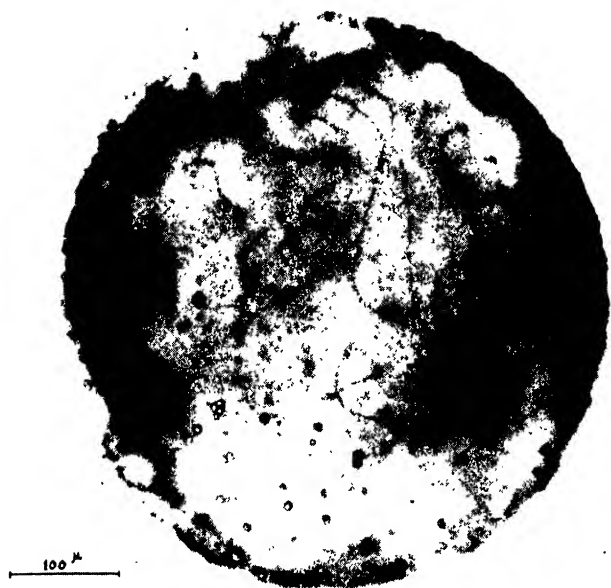


PLATE 6 (For description see facing page).

# FIBER PROTEIN STRUCTURE IN CHROMOSOMES AND RELATED INVESTIGATIONS ON PROTEIN FIBERS

By Daniel Mazia\*

*University of Missouri, Columbia, Missouri*

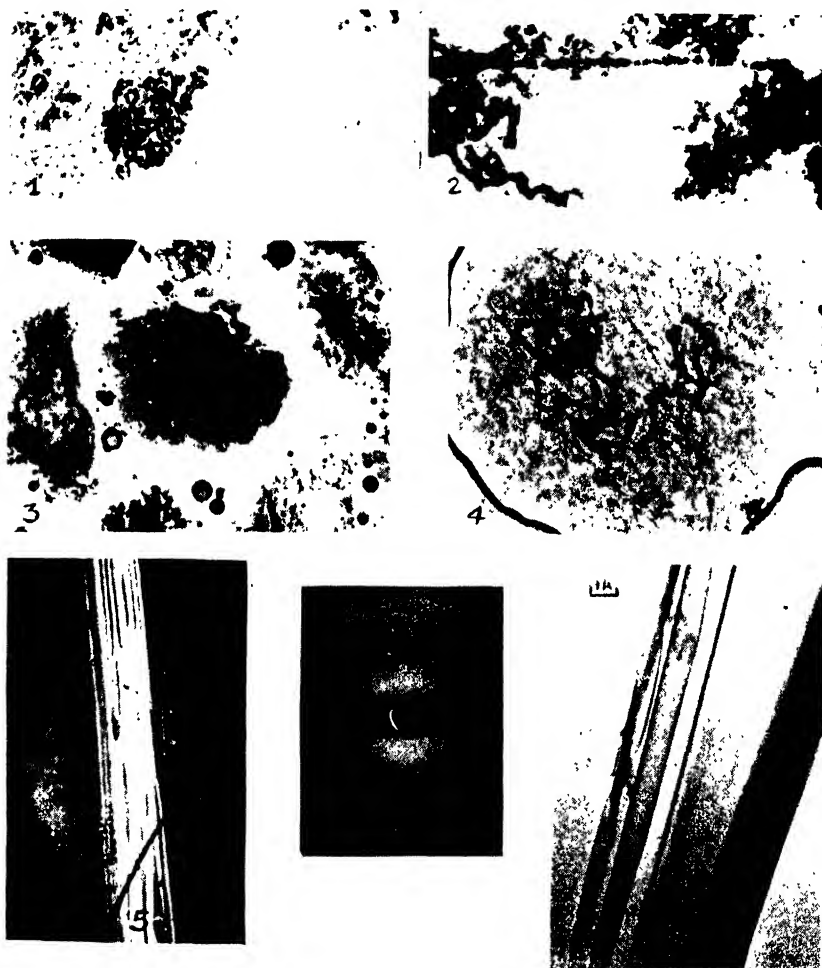
The numerous problems of chromosome function fall into two major groups: (a) those concerning the chemical composition of the chromosome and directed toward an ultimate comprehension of the nature of genic material and (b) those concerning the molecular architecture of the chromosomes as expressed in their visible and mechanical properties, their movements and reproduction in mitosis and meiosis, and the phenomena of breakage and recombination. This paper is concerned with the latter question at its most elementary level, the question of the intermolecular forces responsible for the organization of proteins and nucleoproteins into the tangible, elastic, "insoluble" body observed under the microscope, and subject to manipulation.<sup>1</sup>

Direct means are not available for a study of this question, nor, because the classes of compounds found in chromosomes are generally studied in solutions, do we have even a clear conception of the forces involved in the realization of structure at this level.

The action of proteolytic enzymes on dipteran salivary gland chromosomes has been studied from this point of view.<sup>2</sup> Once it was shown that the nucleic acids, as such, did not seem to be essential to the maintenance of the form and material continuity of salivary gland chromosomes, it was possible, applying knowledge of the peptide specificity of certain proteases,<sup>3</sup> to consider the protein constitution of the chromosomes in relation to their structure. The basic observation is that of Caspersson<sup>4</sup> that trypsin effects complete dissolution of the chromosomes, implying a continuous structure of proteins containing basic amino acids. The action of crystalline pepsin, shown in FIGURES 1 and 2, presents a different picture. Represented in FIGURE 1 is the action of crystalline pepsin (5 mg. per ml. at pH 1.5 for 15 minutes) on chromosomes in the whole freshly dissected gland of *Drosophila melanogaster*. The control, FIGURE 2, was exposed to HCl of pH 1.5 alone for the same time. The effect of enzyme is to cause a drastic shrinkage of the chromosomes to less than half of their original volume. The loss of material is largely in the interband regions, these virtually disappearing as the bands pull together. Some of the digested material is also contained in the bands, which become narrower. The general impression is that the enzyme has not affected the staining desoxyribonucleoprotein of the chromosomes, but has removed another component which is normally evident in the interband regions, a protein which contains little or no nucleic acid, but which occupies the larger part of the chromosome volume. It is to be noted that the removal of this large volume of material does not cause any breaks

\* The author wishes to acknowledge the assistance of the American Cancer Society, through the Committee on Growth of the National Research Council, which made much of this work possible. The unpublished portions of the investigations summarized here will be published in collaboration with Dr. Teru Hayashi, now of Columbia University, and Mr. Kenneth Yudowith, Department of Physics, University of Missouri.

in the continuity of the chromosome. It remains a miniature version of the original structure, in fact, approaching "normal" chromosomes in size. The



FIGURES 1-7

FIGURE 1. Chromosomes from gland digested with pepsin (5 mg. crystalline pepsin per ml., pH 1.5, 25°C, 15 minutes). Acetocarmine.

FIGURE 2. Pepsin control. Gland treated with HCl alone for 15 minutes. Time, temperature, and pH same as in FIGURE 1.

FIGURE 3. Control to cathepsin experiment. Gland immersed for 30 hours in buffer pH 3.5 containing 1 mg. per ml. of NaCN.

FIGURE 4. Chromosomes from gland treated with cathepsin solution (Activity described in text) 30 hours at 35°C.

FIGURE 5. Polarized light view of surface-compressed albumin fiber.

FIGURE 6. Wide-angle X-ray diffraction of albumin fibers. Ni-filtered Cu K $\alpha$  radiation (wavelength 1.54 Å). Camera radius 5.377 cm.

FIGURE 7. Electron microscope view of portion of albumin fiber not cast.

experiment is easily interpreted on the basis of the peptide specificity of the enzyme. Pepsin is specific for peptide linkages involving an acidic amino



acid residue and is inhibited by the proximity of a basic group. It would not be expected, therefore, to digest many linkages in a protein of the histone type, but to affect proteins with neutral or acidic isoelectric points. It is evident, therefore, that the salivary gland chromosomes possess a dual composition, a continuous structure of histone-like protein carrying the nucleic acid running through a continuous body of relatively acidic proteins containing insufficient nucleic acid to stain appreciably with basic stains. The nucleoprotein portion is obviously concentrated in the bands and runs through the interband region in the form of strands. The acid protein is more concentrated in the interband regions, though accounting for a considerable part of the band volume as well. The nucleoprotein represents a continuous architectural framework, since removal of the other component does not interrupt the typical structure. Whether the acid-protein constituent has a parallel structural significance cannot be determined enzymatically, since trypsin, which digests nucleohistone, also affects most acid proteins.

Extending this enzymatic "dissection," attempts were made to digest the salivary chromosomes with various intracellular proteases in the crude form of tissue autolysates. The crude extracts proving remarkably ineffective, a purified cathepsin<sup>5</sup> preparation was tested, and again, in spite of variations of time, concentration, pH, and activators, the chromosome structure appeared unaltered. FIGURE 3 shows a chromosome from control gland, treated for 24 hours at 35°C with buffer of pH 3.5 containing activator but no cathepsin. FIGURE 5 shows chromosomes treated at the same temperature for the same time with cathepsin solution ( $52 \times 10^{-4}$  Hb units per ml.<sup>5</sup>). Not only are the chromosomes not digested, but the enzyme-treated glands make a superior preparation from the cytological standpoint, because the enzyme has apparently digested nuclear and cytoplasmic proteins that normally interfere with the spreading of the chromosomes after such long treatments. Since the experiments with trypsin and pepsin, as well as chemical extractions<sup>6</sup> and ultraviolet absorption data<sup>7</sup> have characterized the classes of protein present in the chromosomes, their digestibility when in solution may be measured quantitatively. The activity of our cathepsin preparation on hemoglobin (as an example of a pepsin-digestible protein) was determined by the standard method.<sup>5</sup> An activity of  $52 \times 10^{-4}$  Hb units per ml. was obtained at 35°. The activity was also tested on a solution of nucleohistone (from thymus) in 1M NaCl, the measure of activity being the amount of nucleic acid rendered soluble in water at pH 6.0. Ninety per cent of the nucleic acid became soluble within 24 hours at 35°C. Since the substrate remained very viscous throughout, the effect of the enzyme was evidently proteolytic, rather than a depolymerization of the nucleic acid. It should be noted that this digestion by cathepsin of both acidic and basic proteins is to be anticipated from Bermann and Fruton's specificity data.<sup>8</sup>

If cathepsin is capable of digesting solutions of the types of proteins which, upon reasonably good evidence, seem to constitute the major portion of the salivary gland chromosome, the problem is to explain why the chromosomes themselves are unaffected. One possibility would be that the chromo-

some proteins are insulated in some way by layers of lipid or nucleic acid,<sup>8</sup> but this seems to be excluded by the positive results obtained with pepsin and trypsin. Another possibility is that the factor of structural organization as well as peptide composition is determining the reaction of the proteins to the enzyme. There is a precedent for this in the observation of Goddard and Michaelis<sup>9</sup> that keratin, which is not digested by trypsin in its original fibrous form, becomes digestible when put into solution by treatment with thioglycolic acid. The physical organization, not the reduction of the S, is the determining factor, for when the S is reoxidized, the protein remains soluble and also remains digestible by trypsin.

The possibility that the chromosomal proteins are in the fibrous state is suggested by every obvious property. Chromosomes are not easily soluble, are elongate and elastic,<sup>1</sup> and resolve microscopically into distinct threads. The term fibrous no longer implies a distinct class of proteins, but merely a type of physical organization, since it has been shown repeatedly that corpuscular proteins may be converted into fibers. In fact a branch of the textile industry has been built around this transformation. Therefore, it is possible to determine whether the digestibility of almost any protein differs when it is in the soluble, corpuscular state and in the fibrous state. Of the methods available for conversion of corpuscular to fibrous proteins, one that seemed feasible under conditions in the cell was chosen: the unfolding of the molecule to form a monolayer of "surface-denatured" protein and the compression of the film into a distinct fiber, as originally described by Devaux. The procedure is analogous to what Kopac, in this monograph, has called the "Devaux effect," except that in our procedure the film was compressed in one dimension by barriers. Fibers of a variety of proteins have been prepared by this method: ovalbumin, histone, hemoglobin, nucleohistone, casein, *etc.* The digestibility of the fibers, as evidenced by visible disintegration, was compared with the digestion as determined by chemical methods. Their behavior toward trypsin and pepsin followed the expectations from their peptide composition. Trypsin digested all the fibers tested. Pepsin digested fibers of hemoglobin and more acid proteins, but had no observable effect on histone or nucleohistone fibers. It could even be shown that when a fiber composed of a mixture of nucleohistone and albumin was treated with pepsin, only the albumin was digested and the fiber underwent shrinkage. With cathepsin, the artificial fibers, just as did the chromosomes, behaved in an anomalous fashion. None of the fibers was digested, even though the protein solutions from which the fibers were prepared were attacked. Therefore, making allowances for the fact that we are dealing with an impure enzyme preparation, the most obvious conclusion is that some structural change involved in the transformation of proteins into the fibrous state renders them resistant to hydrolysis by cathepsin. In so far as conclusions can be drawn from negative results, the parallel behavior of chromosomes and artificial fibers of types of proteins composing chromosomes is taken to indicate the possibility that their basic structural organization is comparable. Further refinement may possibly make digestibility by cathepsin a test for proteins in the fibrous state. At the very least, this

evidence adds a somewhat more direct argument to the inference already drawn by other workers, summarized by Bateman,<sup>10</sup> that the physical properties of chromosomes derive from the fibrous state of proteins.

Most of our knowledge of the structure of the protein fibers is based on work with natural fibers of relatively simple and biologically inactive proteins. Assuming the possible validity of the parallelism between the molecular structure of chromosomes and of protein and nucleoprotein fibers prepared by the technique described, the structure and biological reactivity of these fibers has been investigated. The results, as will be seen, give some insight into the biological possibilities of the fibrous state of proteins independently of the application to the special case of chromosomes.

Microscopically, the surface-compressed fibers appear as bundles of fine, refractile fibrils, even under the highest magnifications of the light microscope. They are highly birefringent, as shown in FIGURE 5, which represents a portion of an ovalbumin fiber. Measurements of birefringence of wet fibers yield a value of 0.002, positive with respect to the fiber axis. This is rather a high value when allowance is made for the water content, and it must be concluded that some element in the structure is oriented. The material lends itself very well to electron microscope observation. FIGURE 7 is a view of a portion of a fiber of ovalbumin. The fibers of other proteins, including nucleoproteins, were essentially similar in appearance. The fibrillar appearance of the fibers under the light microscope turns out to be an illusion resulting from folding. The fibers, which were prepared by compressing, monomolecular sheets, remain sheets. The striations are the product of multiple thicknesses of the unit sheet, which is very thin. Whether it is actually monomolecular cannot be ascertained, since there is no means of measurement of thickness. Nothing in the observations excludes the possibility.

Since the folded sheets do show positive birefringence, their X-ray diffraction was investigated, with a view to determining the type of transformation effected by the unfolding at the surface. Facilities only for wide-angle diffraction were available, but these were expected to be adequate, since something analogous to Astbury's keratin patterns was anticipated. The actual results of an exposure of a bundle of parallel ovalbumin fibers (wavelength 1.54 Å, camera radius 5.377 cm.) appear in FIGURE 6. The rings correspond to spacings of 3.2 and 4.2 Ångstroms, with a suggestion of a spacing of 10-12 Å, *but there is no evidence of orientation*. These roughly correspond to the beta keratin spacings which have also been observed in denatured albumin. The best interpretation that seems possible from these data is that the fibrous structure does not depend upon a grid of polypeptide chains parallel to the fiber axis. The evidence of the spacings described would indicate that the chains are at least in part extended, and the extended chains folded in a two-dimensional pattern that has no compulsory relation to the fiber axis and, therefore, is potentially specific. It is easiest to imagine the fiber as a grid of flattened molecules, the orientation of whole molecules being responsible for the orientation observed with polarized light. If this is the case, evidence of orientation with large spacings should be ex-

pected in small-angle diffraction measurements. Such measurements have been undertaken.

With respect to the general problem of fiber structure, probably the main significance of these observations derives from the fact that proteins may form strong, elastic stable fibers by linkage of two-dimensionalized individual molecules without the drastic arrangements that would be required by the keratin picture. It is of further interest that the molecular units within the fiber can be low-molecular weight proteins and that the fibers may have a mixed composition. There need not, therefore, be any chemical difference, but only a difference in physical organization, between "structure" proteins (a term usually limited to highly asymmetric large molecules) and the soluble proteins of the cell. With respect to the immediate problem of chromosome structure, it is evident that the type of fiber structure visualized corresponds closely to the requirements of the simplest hypothesis of chromosome duplication, namely, that the original molecules provide the pattern upon which the daughter molecules are synthesized. This cannot easily be conceived except in terms of an essentially two-dimensional molecular structure. The picture of the chromosome thread as a microscopically and mechanically fibrous structure that is, submicroscopically, a folded film meets these requirements. The fact that this is true, of course, does not constitute proof of the hypothesis, but the hypothesis does establish the physical plausibility of concepts of chromosome duplications that have seemed, to some, to be required by the facts of genetics and cytology.

Since the proposed structure depends on establishment of a fibrous condition by "surface denaturation" (and any interface between an aqueous and non-aqueous phase is adequate), the difficulty presents itself at once that such a structure might be incompatible with the specific reactions, which are believed to take place in the chromosome, dependent on the "native" state of the enzymes involved. Even assuming that "surface denaturation" does not necessarily lead to loss of specificity, an assumption for which some evidence has been available,<sup>11</sup> interaction of constituents of these rather solid structures might present problems not ordinarily visualized in the kinetic studies of enzyme action in solution. Yet, such interaction presumably takes place in the chromosome, expressed as "position effects" and similar phenomena. It has proved to be relatively simple to investigate the possibility and the properties of enzyme-substrate reactions when both are contained in a solid fibrous structure.

By using a mixture of proteins as starting material, one may prepare films and fibers containing the proteins in approximately their original proportions. The composition of the film may be verified by determining with the surface balance the area occupied by a given amount of mixed protein and comparing this with the expected area obtained by separate spreading of the components of the mixture. In the present case, mixtures of crystalline pepsin and crystalline ovalbumin were spread over buffer of pH 4.2, where the enzyme is inactive and spreading is satisfactory. The basic facts that the enzyme has not lost its activity and that it may react with the substrate when both are contained in the solid phase of a fiber are demonstrated visu-

ally by placing the complex fiber in a medium of pH 1.5. Under the microscope, the fiber is observed to digest itself and to disappear in a short time. For example, a fiber with an albumin-pepsin ratio of 20 to 1 disappears in about one minute at pH 1.5. This microscopic method of estimating enzyme activity from the autolysis time becomes fairly reproducible, and it is possible to obtain a pH optimum curve, a measure of the relation between proportions of enzyme and substrate, to observe that the system is susceptible to heat denaturation, *etc.*

However, for assurance that this visible disintegration of the fibers actually is the result of hydrolysis of peptide linkages, it is necessary to employ a chemical method. Pepsin albumin fibers were collected in quantity,

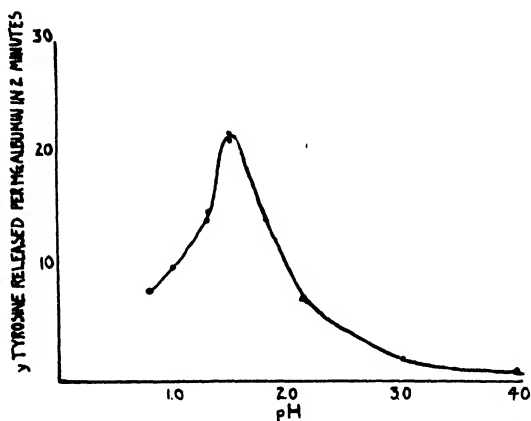


FIGURE 8. The pH dependence of the digestion of an albumin-pepsin film. Enzyme-substrate ratio 20:1. Temperature 30°C. Ordinates: gamma "tyrosine color equivalents" produced per mg. of substrate in two minutes. (Tyrosine color equivalent is the amount of digestion products giving color with the Folin-Ciocalteu reagent, expressed in terms of the amount of tyrosine given the same color value.)

and weighed out in 1 mg. samples, which were exposed to 0.3 ml. of the desired buffer for the experimental period. The reaction was stopped by trichloroacetic acid, and filtration and colorimetric determinations of split products yielding color with the Folin-Ciocalteu reagent followed on a micro scale the Anson method.<sup>12</sup>

There is no doubt that the pepsin digests peptide linkages in the albumin when both are contained in a surface-compressed fiber. FIGURE 8 indicates both that peptides soluble in trichloroacetic are produced and that the pepsin action has about the same pH optimum as when acting in solution.

The rate of digestion as the proportion of substrate to enzyme is varied is shown in FIGURE 9. It is evident that the initial rate of action is high. This is the part of the reaction that presumably takes place in the solid fiber. The later slow reaction probably reflects the action of the pepsin liberated from the fiber on the fragments remaining after visible disintegration. It is apparent, also, that digestion takes place even when the ratio of substrate

to enzyme is as high as 80-1. From the standpoint of the problem of enzyme reactions in solid phase systems—a question of importance to the cell physiologist independent of the applicability of this particular system to the special case of chromosomes—these data raise some interesting questions. The geometry of the system is such that the unfolded molecules would seem to be in contact with each other only at their margins. Apparently, an enzyme can activate a substrate molecule in this relationship. It does not seem likely that an enzyme molecule in the film can be in contact with as many as 40 substrate molecules. It would be anticipated that the first part of the reaction would, at most, involve the action of the enzyme on its few neighboring substrate molecules, the enzyme then being liberated into the medium leaving a continuous fiber with holes in it. Actually, the fiber

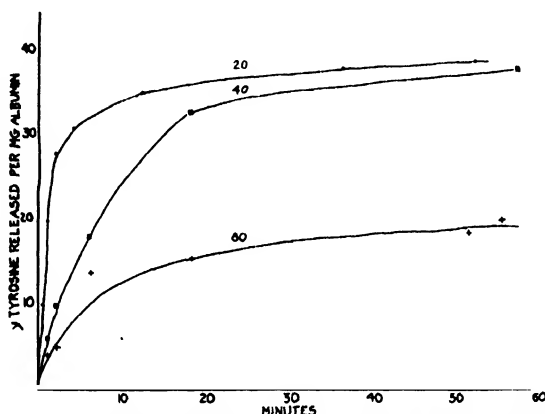


FIGURE 9. Rate of digestion of albumin-pepsin fibers as a function of substrate-enzyme ratio (numbers over curves). Ratios represent absolute amounts of crystalline albumin and crystalline pepsin. Ordinates: tyrosine color equivalents of split-products soluble in 6 per cent trichloroacetic acid.

disintegrates early in the reaction and the analytical data indicate that a large proportion of the ultimate products is released in the early rapid phase. It cannot be postulated that the liberated enzyme is, after digesting its way out of the film, acting from the bulk medium, because, as will be seen in FIGURE 10, the action of dissolved pepsin is much slower than that observed in the complex fibers. One possible explanation is that the liberated pepsin is trapped in the layers of the fiber mass and, therefore, acts from small volumes of high "concentration." This is to be tested by observing the reaction in very thin films, employing the electron microscope. Another possible explanation is that the pepsin is not liberated into the liquid phase, but works its way around an ever-widening hole in the film, always attached at some points. Finally, the work of Rothen<sup>13</sup> renders plausible the most obvious explanation of these results: that the enzyme need not be in contact with substrate molecule. In fact, the pepsin-albumin fiber system, composed as it is of closely linked protein molecules, would seem to make less demands on the "energy continuum" concept of long-range action than does

the Rothen experiment on the action of trypsin through layers of Formvar. The crudity of these concepts and the fact that one finds it impossible to apply to this system the very term "concentration," and yet has no substitute, merely illustrate how badly cell physiologists, who have to deal with the gross as well as the microheterogeneity of the cell, require the development of a chemistry of reactions in structurally restricted systems.

FIGURE 10 shows how the structure of the pepsin-albumin system may affect the rate of reaction. In these experiments, the amount of substrate was the same (1.0 mg.) and the total volume of reaction mixture was the same (0.3 ml.). The amount of enzyme was greater in the case where pepsin in solution was acting on fibrous albumin. It is evident that the reaction proceeds much faster when both enzyme and substrate are contained in the fiber.

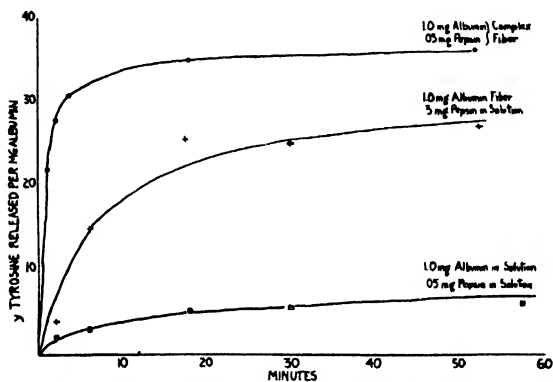


FIGURE 10. Rate of digestion of albumin-pepsin fibers compared with digestion of albumin fibers by dissolved pepsin and dissolved heat-denatured albumin by dissolved pepsin. Ordinates: tyrosine color equivalents of split products soluble in 6 per cent trichloroacetic acid.

Thus, far from causing loss of activity, transformation of the enzyme-substrate system from the soluble to the fibrous state actually increases the rate of activity. One might predict that this would be the case because the reactants are "concentrated" in the fiber. One might also predict the reverse result, not only because of the factor of surface denaturation, but because of the limited mobilities in the solid phase. In any event, the rate of activity would not be predictable from information as to the amount of enzyme and substrate and the volume of the system. The configurational factor would have to be known. These observations permit us to investigate the possibilities of a fibrous but, essentially, two-dimensional molecular structure for chromosomes, with the assurance that enzyme reactions can take place within such a system and at a high rate.

Since the earlier work on the activity of surface denatured proteins, the question has always been raised whether the specific activity found may be attributed to adsorbed unspread protein or partially spread protein in the film. As to the latter, it is now the opinion of some surface chemists<sup>14</sup> that the spreading is an all-or-none phenomenon, the varying "thicknesses" re-

ported in earlier papers representing merely varying proportions of material going into the film. As for adsorbed pepsin, we were unable to find evidence of it in experiments where pepsin solution was injected under an albumin film. After the thorough washing to which all our fibers were subjected, the albumin fibers remained inactive. The discussion of this question is rather involved, and will be presented in another place.<sup>15</sup> Here, it need only be said that we have found no evidence contrary to the conclusion that in the complex films and fibers the pepsin molecules are unfolded, and much evidence in favor of it.

Of the distinctive properties of chromosomes which seem to derive from their structural organization rather than from their composition, perhaps the most workable is their sensitivity to radiations. X-ray doses of a few to a few hundred r units cause chromosome breakage and mutations, while *in vitro* experiments on proteins and nucleic acids show that tens or hundreds of thousands of r units are required to effect a measurable chemical change. The experiments of Dale,<sup>16</sup> showing effects of small doses, presuppose a dilute and pure solution of enzyme protein, a condition that would hardly be expected in the cell. It seemed possible that the sensitivity of chromosomes might be due to the susceptibility of intermolecular linkages involved in their mechanical structure rather than intramolecular configuration. To test this possibility on a simple system, we have irradiated albumin and pepsin-albumin films and determined their mechanical properties as expressed by force-area curves. The results were negative up to 6000 r. The compressibility of the film remained unaltered. The next possibility was that radiation might affect the enzyme-substrate linkage or, returning to the original concept, the enzyme itself. It is a very simple matter to irradiate a pepsin-albumin film, compress it into a fiber, and estimate its activity in terms of the time required for visible disintegration. These estimations become quite reproducible with experience, although obviously they must be checked by the quantitative method.

The results obtained in preliminary experiments do indicate a close correlation between the structural configuration and the sensitivity to radiation. In the control experiments, the 20:1 albumin pepsin solution (pH 4.2, total protein 5 mg. per ml.) was given as much as 6000 r (Al filtered Cu radiation) and then spread on buffer of pH 4.2. The film was compressed into a fiber and the digestion determined by applying HCl of pH 1.5. The digestion time of unirradiated material ranged from 45 seconds to 70 seconds. The digestion time of the irradiated material fell within the same range. These results are not surprising, since very much higher doses are generally required to cause enzyme inactivation in relatively concentrated solutions and in the presence of a large amount of other protein. When the same mixture is first spread, then irradiated, and finally compressed and tested for activity, the effectiveness of the radiation is very different. After a dose of 390 r (the dosage data are approximate, based on a previous calibration of this tube), the fibers no longer digested at all in HCl of pH 1.5. After a dose of 140 r, the digestion time was 10 minutes as compared with a control (unirradiated) of 45 to 70 seconds. After 70 r, the digestion time was 4-5



minutes. It is evident that even with this crude method of measurement, the effects of a dose as small as 70 r were evident outside the possibility of error.

Several interpretations of the greatly increased sensitivity resulting from spreading the protein are possible. One is that the pepsin-albumin intermolecular linkages are altered and cannot reform (so far, these effects have all appeared to be irreversible). A second possibility is that, after surface denaturation, a relatively small amount of energy is required to complete denaturation to the point of loss of specificity, a possibility that we are testing in a study of heat denaturation. A third possibility centers around the increased exposure of the unfolded molecules to irradiation products in the medium. This system may offer an interesting tool for the study of irradiation effects on proteins. Obviously, the results conform to the possibility suggested by the earlier part of the investigation, that of the essentially sheet-like structure of the chromosome fiber.

Summarizing, this paper is not presented as a hypothesis of chromosome structure. Such a hypothesis would be useful in cytological and genetic thinking only when it was based on more direct work on the molecular structure of chromosomes themselves. Lacking means for such direct observation, we can only explore those possibilities which conform to existing cytogenetic and biochemical information and which may reveal those properties of chromosomal constituents which emerge when those constituents are organized in a form that at least parallels the gross mechanical properties of the chromosomes.

### *Summary*

(1) The action of trypsin, pepsin, and cathepsin on salivary gland chromosomes is described. The chromosomes are not digestible by a cathepsin preparation which is quite active against dissolved protein and nucleoprotein substrates.

(2) The non-digestibility by cathepsin seems to depend on the fibrous organization of the chromosomal proteins. Proteins and nucleoproteins which are digested in solution become indigestible after conversion to the fibrous condition by compression of monolayers.

(3) The structure of surface-compressed protein fibers has been investigated. The birefringent fibers, which appear to be composed of fine fibrils, on electron microscopic examination, turn out to be highly folded thin sheets, possibly monomolecular. The fibers, in X-ray diffraction measurements, give the spacings of the beta keratin system but no pattern indicating orientation. It is suggested that the fibers are composed of chains or grids of linked molecules, a pattern of polypeptide chain folding within the molecule being preserved.

(4) Fibers composed of pepsin and albumin are highly active enzymatically. The activity is higher than that for the same amounts of enzyme and substrate contained in solution in the same volume.

(5) The complex films of pepsin and albumin may be inactivated by small doses of X-radiation which have no effect on comparable pepsin-albumin solutions.

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### Discussion of the Paper

DR. KURT G. STERN (*Polytechnic Institute of Brooklyn, Brooklyn, N. Y.*): Interesting and ingenious as the experiments here reported by Dr. Mazia are, it is difficult to see how they could throw light on the fine structure of chromosomes. Even fibers prepared from desoxyribonucleoprotein would tend to oversimplify the picture which the cytologists have developed for mitotic chromosomes. Dr. Mazia's experiments suffer from the same objection as all other model experiments of this general type: they tend to explain little and to lure those interested in the actual biological structures into drawing untenable analogies. A case in point are the recent model experiments where "nerve action potentials" were elicited by adding acetylcholine to model systems composed of oil-water interfaces.

DR. D. MAZIA: As a biologist who has been quite actively interested in "the actual biological structures," I should like to emphasize that there was no intention here of presenting a model of a chromosome in the sense of a working imitation. I agree with Dr. Stern on the essential sterility of such an approach. But, just as he has so fruitfully studied *in vitro* the properties of nucleoproteins extracted from nuclei, hoping to gain insight into the nature of gene material, we have examined the structural implications of both chromosomal nucleoproteins and other proteins hoping to learn something about how these materials may be organized at the supramolecular level into the tangible chromosomes we have been studying for a long time. We have done quite a bit of work on desoxyribonucleoproteins (see Ref. 15) and find that the generalizations made in this paper with regard to protein fiber structure apply to them.

We have dealt with non-nucleoproteins as well, because, as pointed out in this paper, they comprise a considerable part of the chromosome material and, as Mirsky has shown recently, have an important part in chromosome structure. This is especially evident in our experiments with pepsin. For the study of activity of proteins in fibrous bodies, we have to use non-nucleo-

proteins, since we have no nucleoprotein, with the possible exception of Avery's "transforming principle," whose activity can be measured. As for misleading biologists, the ideas presented here—and I think anyone familiar with the literature of genetics will agree with me—have been part of the theoretical thinking of geneticists for a long time. Our contribution has been only to demonstrate, if we are correct, that these ideas, particularly that of the two-dimensional configuration of self-duplicating units, are chemically possible. In brief, we are interested in the problem of how the materials which Dr. Stern and others study in the test-tube can become chromosomes in the cells. Lacking direct means of approach, we have explored the possibilities of fibrous organization and tested these by parallel observations on artificial fibers and on chromosomes. If one must use the term "model" at all, our fibers have been used as models of a *kind* of protein organization whose properties may be applicable to the chromosome. This is quite different from a model of a chromosome itself.

DR. ANNA GOLDFEDER (*New York University, New York, N. Y.*): The question arose as to under what conditions the X-radiations were applied to the pepsin albumin fibers and to the various substrates.

In my tissue culture studies, I found that when tissue fragments were left in a nutritive medium of chick embryo extract, chick plasma, and tyrode solution, a lesser dose of X-radiation was required to prevent proliferation than when the fragments were removed from the medium immediately after radiation, washed in tyrode solution, and transferred into a fresh medium. Moreover, when the components of the nutritive medium were irradiated with the same dose separately and later used for cultivation, no apparent effect on the growth of the tissue was observed. Consequently, it might be concluded that some toxic substances are produced during radiation which can be removed by washing the irradiated tissue fragments in a physiological solution.

The observations made by Dr. Mazia on enzyme inactivation are in general agreement with those made by other investigators, namely, that in order to inactivate enzymatic systems, much higher doses of radiation are required than to produce the same effect on chromosomes.

DR. MAZIA: We used 150 kV, Al-filtered, tungsten radiation in these experiments. The films were 20 cm. from the target and radiation was delivered at 13 r per minute. The experiments described by Dr. Goldfeder are very suggestive to workers on chromosomes, because the emphasis on "hit" theories may have overshadowed important indirect effects. The point of emphasis of the experiments on the pepsin-albumin system is that we were able to observe considerable inactivation of the system by doses of less than 100 r. We plan, in this work, to investigate the possibility that the sensitivity of the system is due to the fact that the protein in the films is unfolded, and, therefore, presents much surface for the effect of radiation products in the medium. This investigation would also bear on the "shielding" effects of the components of a mixed system. The pepsin is present in small proportion, but the configuration is such that there is no other protein between it and the aqueous medium.

DR. C. G. MACKENZIE (*Cornell University Medical College New York, N. Y.*): Dr. Mazia, himself, has made it quite clear that the results he has obtained with protein fibers cannot be applied without reservation to chromosomes. Certainly, these experiments represent the gathering of information that is essential for a rational attack on chromosome chemistry, however much more complicated that may prove to be in the light of subsequent work. Dr. Mazia's studies are, therefore, important and stimulating as experiments on chromosome models. But they are of even greater importance in their own right.

Despite the advances resulting from the study of enzymes and substrates in solution, in a homogeneously dispersed system, I am sure all of us are aware of the limitations imposed by such conditions when the results are applied to living systems. In living cells, containing as they do diversified formed elements, reactions must take place not only between molecules in solution but also between molecules and molecular aggregates and between the molecules contained in non-homogeneous solids, that is to say: between the different species of molecules within the formed elements. It is difficult to believe that such is not the case.

Consequently, the fibers studied by Dr. Mazia are far from being just models of chromosomes. They are models of something simpler and, perhaps at our present stage of technical development, of something even more important. They are models of themselves, *of an enzyme and its protein substrate in a solid state*. We now have available a technique for studying the reaction kinetics of such solid enzyme-substrate systems. That, at this higher level of organization, an enzyme-substrate system may exhibit reaction kinetics remarkably different from those it manifests in solution, has already been demonstrated by Dr. Mazia.

I believe that this work represents a revolutionary advance in biochemistry, one that will greatly extend our knowledge of the chemistry of living matter, one that will affect the work of all of us in future years.

# PITFALLS IN HISTOCHEMISTRY\*

By George Gomori

*The Department of Medicine, The University of Chicago, Chicago, Illinois*

Although there were a few successful attempts at the chemical identification of microscopic tissue structures as far back as 80 years ago, histochemistry as a systematic science is relatively young. Many of its methods are still imperfect or not sufficiently standardized, the chemical background and meaning of many staining reactions are poorly understood, and the criteria of correct technique and of proper interpretation are not clearly established. It is readily understandable, therefore, that under such conditions some of the methods or the conclusions drawn from the results obtained do not stand up against rigorous criticism. I should like to illuminate this point in connection with four different techniques. They were chosen because loose interpretation of their results has led to conclusions which do not appear to be warranted.

## 1.

The granules of the enterochromaffin cells (EC) show a number of interesting chemical features which have invited considerable speculation regarding their chemical composition. Their most important chemical reactions are the following:

(1) The chromaffin reaction, shared by the cells of the adrenal medulla, consists in a brown staining of the granules by dichromates. This shade is due to both the reduction of dichromate to  $\text{CrO}_2$  and the formation of colored oxidation products.<sup>1</sup> A similar reaction occurs if dichromate is added to an aqueous solution of adrenalin. Using this observation as a starting point, Verne<sup>2</sup> took crystals of a number of related compounds and observed the formation of colors on the addition of dichromate. He found that all o- and p-diphenols and aminophenols are chromaffin; *i.e.*, they produce dark brown pigments when treated with dichromate. On the contrary, m-diphenols and -aminophenols gave no reaction.

(2) The argentaffin reaction consists in the reduction of an ammoniacal  $\text{AgNO}_3$  solution to metallic Ag, with blackening of the granules. It should be made clear, as stressed by Cordier<sup>3</sup> and Hamperl,<sup>4</sup> that the argentaffin reaction is fundamentally different from all other silver impregnations. In the argentaffin reaction, it is a substance contained in the granules themselves that achieves the reduction (Masson-Fontana technique),<sup>5</sup> whereas, in other silver impregnations an extra reducer, applied before or after the silver solution, is responsible for it. Misuse of the term "argentaffin" has resulted in considerable confusion. The argentaffin reaction, if performed correctly, possesses a marked chemical specificity, while silver impregnations in general are not specific in the chemical sense of the word, no matter how selective they may be for certain morphologic structures. Therefore,

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the only silver stains that can be considered selective for argentaffin substances are those which utilize the reducing power of those substances without employing a secondary reducer. Before specificity for any other type of stain can be claimed, it must be shown conclusively by the use of consecutive serial sections that it stains exactly the same cells as the Masson-Fontana technique.

The number of known true argentaffin substances in paraffin-embedded tissues is very limited and includes, besides polyphenols, urates and some poorly known aldehydes. Adrenalin is argentaffin only if the silver solution is applied to fresh tissue. There is no fixative known that will preserve it. The granules of the EC are preserved by all formalin-containing aqueous fixatives but dissolved by all formalin-free aqueous fluids and by alcoholic ones, whether or not they contain formalin.<sup>6, 7</sup> This is an important point, since Dawson<sup>8, 9</sup> and Sharples<sup>10, 11</sup> have used the Bodian method, which involves fixation in an alcoholic medium and the application of a secondary reducer, for the demonstration of the EC. Now, after Bodian's fixative, all the typical reactions of the EC become entirely negative. It is the more remarkable that, in spite of this, Bodian's method will pick out exactly the same cells as the specific stains in the colon and in the small intestine, although in the stomach a number of additional cells are stained. The significance of this fact is not clear, and further investigation is needed for its elucidation. The Popoff<sup>12</sup> stain, on which an original theory on the nature and physiologic role of the EC is based, is entirely nonspecific.

(3) The azo-reaction<sup>13, 14</sup> consists in the production of brilliant reddish azo-dyes when the section is immersed into the alkalinized solution of a diazotized amine. This reaction is almost absolutely specific for phenols, and the shade produced depends partly on the nature of the amine used and partly on the complexity of the phenol. The simpler the phenol, the yellower the shade; the more complex, the more purplish the shade.

(4) The indophenol reaction<sup>15</sup> consists in the production of bluish indophenol dyes when the section is treated with dimethyl-p-phenylene-diamine and an oxidant such as NaOCl. In my experience, this reaction is of very limited practical usefulness, at least in vertebrate material, because the shade produced is too pale.

There are a number of other more or less specific reactions described, part of which are poorly understood chemically. Since they are of minor importance, they will not be discussed here.

On the basis of the reactions mentioned, Lison and his coworkers<sup>13, 15</sup> drew the following conclusions: (a) the EC contain some o- or p-diphenol; (b) since the azo-dyes formed are insensitive to alkali, the coupling must have occurred in o-position, which in turn means that the p-position is not free, because whenever that position is free, coupling invariably occurs there; (c) the group attached at the p-position must be relatively simple because the shade of the azo-dyes formed is yellowish. A catechol compound, with a short side chain in p-position, would therefore show all the typical reactions of the EC.

The ideas of the Lison group have been universally accepted and un-

challenged up to this date. There are three weak points in Lison's reasonings, however, which seem to have escaped the attention of subsequent workers.

First of all, the mere fact that the indophenol reaction is positive speaks against a p-substituted phenol, since indophenol dyes, as a rule, do not form unless the p-position is free. Second, derivatives of catechol and hydroquinone, such as adrenalin and homogentisic acid, respectively, are far stronger reducing agents than the granules of the EC. They will reduce an ammoniacal silver solution almost instantly, whereas the granules require several hours. Third, the rule that p-coupled azo-dyes change their shade in alkaline solutions, while those of the o-coupled variety do not, is one to which there are numerous exceptions. Certainly, no positive conclusions can be drawn from the fact that the shade of an azo-dye is resistant to alkali. A number of observations were made recently which cast considerable doubt on the correctness of Lison's ideas.

If Gibbs's reaction,<sup>16</sup> which consists in the formation of blue indophenol dyes when a phenol is added to the alkalinized solution of 2,6-dichloroquinone-chloroimine, is applied to sections, a distinct reaction is observed in the granules of the EC. This reaction is negative with catechol and also with any p-substituted phenol.

It can be shown that, under the conditions of histologic technique, the typical reactions of phenols are not the same as they are in the test tube. This difference, according to Coujard,<sup>17</sup> is due to the fact that phenols condense with formaldehyde to form insoluble bakelite-like resins. Therefore, he suggests that, in place of simple test-tube experiments, the conditions of histochemical technique should be duplicated by dissolving the substance in question in serum, writing a mark on a slide, and treating the slide subsequently with all the reagents used in the histologic routine. From the results of reactions applied to such slides, valid histochemical conclusions can be drawn. By the use of this technique, I was surprised to find that resorcinol, although not chromaffin in the test tube, is made just as chromaffin as is catechol. Furthermore, catechol and hydroquinone reduce an alkaline silver nitrate solution within a few minutes, while for resorcinol it takes several hours to do so. The Gibbs reaction, too, is quite intense with resorcinol but negative or negligibly positive with both catechol and hydroquinone. The most striking results, however, are seen with the azo-reaction. Certain diazotized amines will produce various shades when coupled with different phenols, and it is quite obvious that the shades obtained with resorcinol are invariably identical with those shown by the enterochromaffin granules, while the shades obtained with catechol and hydroquinone are utterly different. Therefore, on the basis of these experiments, it would seem that the histochemical reactions of the EC are due to some derivative of resorcinol and not of catechol as it has been believed. This would be quite interesting, since no resorcinol derivative has been known so far to be of physiologic importance in animals.

One more point should be mentioned briefly. Jacobson of Cambridge, England, has adduced excellent evidence<sup>18, 19</sup> to the effect that both EC and

tumors arising from them contain some pteridine. He also makes the statement, however, that the typical reactions of the EC may be due to the presence of pteridines. This is definitely not the case, as can be shown by Coujard's technique. Pteridine does not give a single one of the typical reactions. I was also unable to confirm Jacobson's other contention, that of the presence of desoxyribose in the EC.

The conclusion is that, contrary to previous ideas, the typical histochemical reactions of the EC are due to the presence of a derivative of resorcinol. The presence of pteridine cannot be demonstrated in them with chemical methods now available. They do not contain desoxyribose.

## II.

The question of the unity or plurality of phosphatases (Ph) has been a moot point for almost 20 years, with numerous champions on both sides of the fence. There can be no doubt that acid and alkaline Ph are two distinctly different enzymes. However, those who support the theory of plurality find that Ph's are different among themselves in 3 more respects: (1) substrate specificity; (2) organ specificity; and (3) specific activation and inhibition effects. The latter two groups often overlap.

As far as substrate specificity is concerned, it has been established beyond doubt that adenosinetriPh, pyroPh, and hexosediPh are enzymes distinctly different from the nonspecific alkaline Ph. However, Forrai<sup>20</sup> thinks that there is a specific sucrosePh and other sugarPh's; Roche and Latreille<sup>21</sup> maintain that the kidney contains, besides glyceroph, a phenylPh; and Reis<sup>22, 23</sup> claims the existence of a 5-nucleotidase.

There are data available to the effect that alkaline Ph's of different organs may actually be different enzymes, as shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti and coworkers<sup>24</sup> and Hommerberg<sup>25</sup> believe that bone Ph is different from renal or hepatic Ph. Bodansky<sup>26</sup> thinks that intestinal Ph can be distinguished from bone or renal Ph. Masayama and Shuto<sup>27</sup> found a Ph in hepatomas, different from the enzyme of the normal liver. Cloetens<sup>28, 29</sup> distinguishes two alkaline Ph's on the basis of their different degrees of activation by Mg. Drill, Annegers, and Ivy<sup>30</sup> find that in jaundice a Ph appears in the plasma, different from the normal enzyme in respect to inactivation by cyanide.

The opinions just mentioned represent but a fraction of the literature. Whether the differences reported should be considered as an indication of the existence of several truly different enzymes or as the results of the admixture of various activators and inhibitors or of differences in technique cannot be decided on the basis of our present knowledge. The majority of the experiments were never repeated and the results neither confirmed nor refuted by others than the original authors.

During the last few years, I have prepared a number of highly active purified alkaline Ph extracts from intestinal mucosa, kidney, and bone. The activity of some of the extracts is over 1000 Bodansky units per mg. of N. These enzymes of various sources are entirely indistinguishable from each other in respect to specificity toward 10 different substrates, pH opti-



mum, activation by Mg. or inhibition by cyanide. To put it more exactly, differences between enzymes of different origin are not more significant than those observed between two random batches of, say, intestinal enzyme. However, the yield per Gm. of tissue may show an individual variation of several 100 per cent.

The question is: Can histochemical technique be utilized as an approach to the solution of the problem? A number of papers answer the question in the affirmative (Glick,<sup>31</sup> Dempsey and Singer,<sup>32</sup> and Dempsey and Deane<sup>33</sup>). Because I feel that some of the inferences drawn from the pictures obtained are of doubtful validity, I should like to point out certain sources of error.

First of all, the histochemical demonstration of Ph is a very crude method as far as the quantitative aspect is concerned. It is exceedingly difficult to judge the intensity of the stain. In the majority of instances, Ph occurs highly concentrated within sharply limited areas. If the reaction is positive, one gets a black spot and, since nothing can be blacker than black, the picture may not change appreciably if the slide is incubated for 12 hours instead of 1 hour, except for a slight peripheral extension of the positive areas and some fuzziness of the outlines. Just how much of this is due to diffusion artifacts and how much to additional genuine reaction would be hard to tell.

The pH level at which the slides are incubated has a profound influence on the pictures obtained. At pH 9 the solubility of Ca phosphate is exceedingly low but rapidly increases with lowering of the pH. A precipitate of Ca phosphate will form only if the production of phosphate ions is fast enough to cause local supersaturation in spite of diffusion. This race between the rates of phosphate production and diffusion results in an "all or none" effect, depending on whether the rate of phosphate production is or is not able to overtake that of diffusion to a point where the solubility product of Ca phosphate is exceeded locally. At pH  $\pm 8.2$ , the picture is much less intense than it is at 9, and below pH 7.2 it becomes impossible to produce any precipitate of Ca phosphate, regardless of the length of incubation, in spite of the fact that alkaline Ph possesses considerable activity in that range.

The rate of phosphate formation depends on the original activity of the tissue, on the pH, on the presence of activators and inhibitors, on the permeability of the tissues to the various substrates, and on many other factors not too well understood. Of these factors, the pH of the incubating solution again deserves special attention. Alkaline Ph has an optimal pH between 8.8 and 9.8, depending on the substrate, and activity rapidly declines with the fall in pH. At pH 7 the activity of alkaline Ph is only 3 to 8 per cent of its maximum, again depending on the substrate. Acid Ph, on the other hand, has an optimum around pH 5, but it still possesses some activity at the neutral point.

The rate of diffusion depends on many factors, such as the thickness of the section, the presence or absence of a protective layer of collodion (and on its thickness), the amount of Ca phosphate precipitate already deposited, the temperature of the solution, and random currents in it. This complexity of the situation explains the fact that sometimes serial sections treated

exactly the same way will show noticeably different pictures among themselves, especially in the lower pH ranges, where Ca phosphate is relatively easily soluble and enzymatic activity is low.

It is hard to obtain uniformly satisfactory pictures around the neutral point and hard to interpret them correctly. Since at pH 7 Ca phosphate will not precipitate, lead ions must be used to trap the phosphate liberated. The lead salts of most ester phosphates are practically insoluble at this pH. An exception is glycerophosphate, the lead salt of which is sufficiently soluble to be used in histochemical technique. Unfortunately, this substrate is far from being optimal for acid Ph, as shown by the erratic results obtained if it is used for the *in vitro* determination of acid Ph activity and by the often unsatisfactory results of the histochemical technique for this enzyme. Another complication is the tendency of lead to be adsorbed on various morphological structures at this pH, causing spurious positive reactions, especially when nucleate is used as a substrate.

For the reasons mentioned, pictures obtained around the neutral point should be evaluated with great caution. Unless localizations obtained with various substrates are distinctly different and the results can be duplicated in a number of different specimens (to rule out the effect of a chance ratio between acid and alkaline Ph in the given case), no positive conclusions should be drawn.

At this point, I should like to mention my own experiments. So far, 11 different substrates were tried in the range around pH 9: glycerophosphate, phenylphosphate, phenolphthaleinphosphate, hexosediphosphate, yeast nucleate, thymonucleate, adenosinetriphosphate, aminoethylphosphate, phosphorylcholine, lecithin, and octanoylphosphate. After thorough examination of several hundred slides, each carrying 5 to 15 different tissues, I felt that the pictures obtained with the various substrates are not sufficiently different from each other to warrant any assumption of the existence of substrate-specific alkaline Ph's. At pH 5, only 3 substrates were tried: glycerophosphate, hexosediphosphate, and yeast nucleate. The results with the latter two were not very satisfactory but were good enough to convince me that the differences were insignificant. The same substrates were tried at pH 7, and the results, as I expected, were combination pictures of the typical distribution of acid and alkaline Ph in various ratios.

As for the attempts at selective inhibition of specific Ph's, inactivation by KCN, acid, formaldehyde, and heat were tried. My impression was that these inhibitors lack specificity, although the inhibition may not be entirely uniform because, as a result of the "all or none" effect, areas of low activity may be knocked out altogether, while areas of high activity remain seemingly uninfluenced. If the kidney happens to contain a much higher concentration of the enzyme than the intestine, pictures like those published by Emmel<sup>34</sup> may be obtained. I obtained a number of them. If the kidney is lower in activity than the intestine, however, the reverse effect maybe produced. I have several examples of this in my collection (FIGURE 1).

In summary, it may be said that, so far, the results of histochemical technique do not offer any proof for the existence in animal tissues of phos-

phatases other than the common alkaline and acid enzymes. In plant tissues, however, Glick seems to have proven the presence of substrate-specific acid Ph's, the localization of which is strikingly different.

### III.

Bennett,<sup>35</sup> in 1939, described a histochemical method for the demonstration of ketosteroids in the adrenal cortex. Subsequently, his method was utilized for the demonstration of steroid hormones in the ovary (Dempsey and Bassett<sup>36</sup>) and the testis (Pollock<sup>37</sup>). The reaction is based on the formation of yellow phenylhydrazones on treatment of frozen sections with phenylhydrazine. It is prevented by extraction of the lipids with suitable solvents or by treatment with semicarbazide. From these facts Bennett



FIGURE 1. Kidney and small intestine of 3 species. Alkaline phosphatase stain. Left to right: dog, rat, rabbit. Upper row: regular phosphatase stain; lower row: N/150 KCN added. In the dog and rabbit, kidney reaction completely abolished while activity in intestine partly preserved; in rat organs, the opposite holds.

drew the conclusion that the reaction is due to the presence of a lipid ketone, since no lipid aldehydes have been detected in the adrenal cortex.

The serial publications of the Feulgen<sup>38, 39, 40</sup> group on plasmalogen, a cyclic acetal which occurs in large amounts in almost all lipid-containing tissues and which on hydrolysis yields plasmal, a mixture of stearic and palmitic aldehydes, must have escaped Bennett's attention.

In 1942, it was shown<sup>41</sup> that the plasmal reaction, produced with Schiff's reagent, shows a localization invariably and exactly identical with that of the phenylhydrazine test. The opinion was voiced that the latter is not specific for ketosteroids at all but demonstrates an overwhelming bulk of plasmal. It is positive for ketosteroids, too, which plasmal, at certain specific sites, may or may not contain in infinitesimal amounts. The following evidence was adduced:

(1) In both plasmal and phenylhydrazone techniques, the use of an oxidizer (or some hydrolytic catalyst) is necessary before the application of the

reagent proper. The intensity of the stain is, within limits, proportional to the length of pretreatment. In general, it appears that phospholipids require less oxidation than simple neutral fats. Without oxidation both reactions are entirely negative. This is understandable in the case of plasmal, since the acetal linkages must be broken first. But there is no reason why it should be necessary in the case of ketosteroids, which ought to give a prompt reaction without any pretreatment. Therefore, the phenylhydrazone reaction can be accepted as a presumptive evidence for the presence of ketosteroids only if it is positive without any oxydative or hydrolytic pretreatment.

(2) Very intense plasmal and phenylhydrazone reactions were obtained in tissues such as necrobiotic tumors of all kinds, tubercles, brain and peripheral nerves (in fact, Verne<sup>42</sup> suggests the plasmal reaction as a stain for myelin sheaths), and even in adipose tissue. None of these tissues are known to contain ketosteroids.

Dempsey and Wislocki,<sup>43</sup> although acknowledging the fact that the phenylhydrazone test is not capable of differentiating between aldehydes and ketosteroids, still feel that it is reasonable to attribute a positive reaction to the hormones or their precursors. Their main argument is that both the plasmal and the phenylhydrazone reaction are positive at all sites where ketosteroids are known to occur. This is, no doubt, true; but it would hold for a simple fat stain just as well, since all sites where steroid hormones occur are sudan-positive. In fact, the reactions in question are actually not much more specific for ketosteroids than any fat stain. Verne<sup>44</sup> has shown that such pure fats as triolein, butter, or lecithin give typical aldehyde reactions after mild oxidation.

On the basis of the foregoing, all specificity of the phenylhydrazone reaction for ketosteroids must be denied. Why these hormones occur at sites rich in phospholipids is a different problem which deserves attention.

#### IV.

Oster and Schlossman have described a histochemical method<sup>45</sup> for the demonstration of amine oxidase. They incubate frozen sections with a buffered solution of tyramine. The formation of aldehyde is shown by the appearance of a purplish-blue shade when the sections are immersed in Schiff's reagent, fuchsin-sulfurous acid. However, since tissues contain various amounts of plasmalogen, which under the influence of mild oxidants, even air, breaks down to form plasmal, an aldehydic compound, it is necessary first to transform plasmal into a non-reacting form to avoid its being mistaken for newly formed aldehyde. This is accomplished in the following way: First, the sections are treated with a 2 per cent solution of  $\text{NaHSO}_3$  at 37°C. for 24 hours. The bisulfite addition compound of plasmal does not react with Schiff's reagent any more, and the sections, after thorough washing, are ready for incubation with tyramine. Identical reactions are obtained when the sections are incubated with tyrosine. The authors explain this by enzymatic decarboxylation followed by oxidation of the amine thus formed. In fact, there is some reaction obtained even in sections incubated

without any substrate added, owing to the enzymatic breakdown of native substrates.

When this method is analyzed critically, its theoretical background shows a number of weak points:

(1) The oxidation of tyramine by amine oxidase usually goes beyond the aldehyde phase unless the aldehyde formed is trapped (*e.g.* by semicarbazide), and the final product is oxyphenylacetic acid.<sup>46, 47</sup> But, even if the process is stopped at the aldehyde stage, it is unlikely that oxyphenylacetaldehyde, being fairly soluble, would remain *in situ*.

(2) The fact that an identical reaction is obtained with tyrosine as a substrate is very hard to understand, unless it is assumed that the rate of amine oxidation is much faster than that of decarboxylation, and that the amine formed is immediately oxidized. Otherwise, it would diffuse out into the medium, and its local concentration would be exceedingly low. It is possible that such a great difference in the velocities of the two reactions exists; but, so far, there is no evidence for it.

(3) The addition compounds between bisulfite and aldehydes, especially of the higher terms, are very unstable and prone to dissociate into aldehyde and bisulfite. It is unlikely that plasmal-bisulfite would stand up against incubation at 37°C. for 24 hours.

To test the validity of these objections, the following experiments were performed:

(1) A guinea-pig kidney mash was incubated with buffered M/50 tyramine for 24 hours, and a control was run without tyramine. The deproteinized filtrates were both Schiff-negative, showing that no aldehyde was formed.

(2) Frozen sections of guinea-pig kidney were treated with bisulfite for 24 hours and washed thoroughly. They were incubated for 24 hours with tyramine and with buffer solution alone. In addition, several sections were immersed for 10 minutes in 2 per cent trichloroacetic acid, thoroughly washed, and incubated with tyramine. After incubation, all sections were immersed in Schiff's reagent, where all of them started to get bluish after about 10 minutes and were quite blue after 45 minutes. Slices incubated with or without tyramine or after trichloroacetic acid treatment did not show any appreciable difference in the intensity of the reaction. Moreover, the distribution of the blue staining areas under the microscope could not be distinguished from the regular pattern of the distribution of plasmal, although the shade was different. It should be remarked here that no reaction is obtained with Oster and Schlossman's technique if liver sections are used,<sup>48</sup> although liver has a high amine oxidase activity. This negative result may be due to the fact that liver contains practically no plasmal.<sup>44</sup>

(3) The color reaction of 4 different aromatic aldehydes with Schiff's reagent was tested. Unfortunately, p-oxyphenylacetaldehyde was not available. The following reactions were obtained: benzaldehyde, purple, shade indistinguishable from that of plasmal; anisaldehyde, definitely more reddish; vanillin, rose-red; p-dimethylaminobenzaldehyde, scarlet. None of the aldehydes showed the blue shade of the sections.

On the basis of these results, it would seem that Oster and Schlossman's

technique does not demonstrate amine oxidase. The blue shade may be due to some alteration in the plasmal molecule, regenerated from its bisulfite compound, but the exact chemistry of this process is not clear. It may have something to do with the pH of the bisulfite solution. A fresh solution of bisulfite has a pH of around 5, but at 37°C. it may turn very acid (pH 2.4 to 2.6) within a few hours. Some solutions, however, for a reason not clearly understood, will show very little lowering of the original pH. This unpredictable behavior of the bisulfite solution may explain the poor reproducibility of the results, admitted by Oster and found by me.

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### *Discussion of the Paper*

H. WAELSCH (*Columbia University and New York State Psychiatric Institute and Hospital*): Results obtained in determining the higher fatty aldehydes (plasmal) with the aid of the Schiff reagent have to be accepted with caution, since it can easily be shown that the age of the preparation and the accompanying lipids influence the values decisively. Lecithin, after 5 recrystallizations as the cadmium complex (phosphorus-choline ratio 1:1),

gave, after 4 days over sodium hydroxide at 15 mm. pressure, a fuchsin color value corresponding to 70 gamma palmitaldehyde; after 8 days, a value of 185 gamma; and, after 14 days, one of 235 gamma per 100 mg. This increase in apparent aldehyde content may be related to an oxidation of the unsaturated fatty acids and may simulate, in a preparation of not well-defined history, a high original aldehyde content.

On the other hand, if one carries out the Feulgen test with synthetic aldehydes or acetals in the presence of lecithin or synthetic non-ionic detergents, the color values may be suppressed down to a few per cent of the control sample.

DR. G. GOMORI: No doubt the age of the tissue is an important factor in the intensity and the extent of both the plasmal and the phenylhydrazone reaction. Frozen sections which are kept floating, unprotected from air for a few days, will become increasingly positive for plasmal. All rancid fats are also extremely strongly positive.

M. A. LESSLER (*Department of Biology, New York University, New York*): There is need for a critical re-evaluation of the specificity of both the plasmal and the Feulgen reactions. Although the same reagent is used in both (leucofuchsin, produced by treatment of basic fuchsin with an excess of sulphurous acid), the Feulgen reaction involves a preliminary mild acid hydrolysis which is not used in the plasmal reaction.

Is it not true that we should interpret the plasmal reaction cautiously, since it is simply an application of the Schiff reaction for aldehydes to tissue sections? This reaction has been shown to be non-specific by Lison (*Bull. d'Hist. Appliquée* 9: 177. 1932) and others. Leucofuchsin can be recolorized on reaction with basic compounds such as the purines, pyridine, and methyl ketones and other naturally occurring intracellular substances such as starch, glycogen, and other polysaccharides. Treatment of the sections with aqueous and then with alcoholic solutions removes certain soluble substances, but even after such extraction there is still present in some cells a residue which will regenerate the color of the leucofuchsin. Therefore, conclusions as to the nature of the residue cannot be made unless other techniques are used for the purpose of eliminating the possibility of several compounds entering into the reaction.

On the other hand, the Feulgen reaction can be controlled by use of sections which are not subjected to the mild acid hydrolysis. These should be completely negative to the leucofuchsin reagent. If the controls stain, they are showing the non-specific plasmal reaction. The sections subjected to the Feulgen reaction should be interpreted only in the light of the controls.

With adequate control, the Feulgen technique is uniformly specific for nuclear material. The interpretation that this technique stains the aldehydes formed by the mild acid hydrolysis of thymonucleic acid is open to some question at present. Stedman and Stedman (*Nature* 152: 267. 1943) and Carr (*Nature* 156: 143. 1945) have raised objections against the specificity of the Feulgen reaction. These have been refuted by Stowell (*Stain Tech.* 21: 137. 1946) and Dodson (*Stain Tech.* 21: 103. 1946).

Interpretations of the Feulgen reaction are of basic importance for an



understanding of the chemistry of tissues. A reinvestigation of the specificity of this reaction is being conducted in our laboratory.

DR. G. GOMORI: The plasmal reaction has a limited specificity but, if it is performed correctly, there is little interference on the part of non-aldehydic substances. Uric acid and saccharides, for instance, do not recolorize leucofuchsin under the conditions of the test. In addition, just as non-hydrolyzed sections can serve as controls for the Feulgen test, sections treated with semicarbazide or, preferably, with phenylhydrazine can be used as controls for the plasmal reaction.

DR. H. STANLEY BENNETT (*Massachusetts Institute of Technology, Cambridge, Mass.*): Since Dr. Gomori has chosen to discuss the phenylhydrazine reaction, which I applied to the adrenal gland, it is appropriate that I comment on his paper.

First, I might stress that I made no claims that the reaction I used was one which demonstrated the presence of ketosteroids. In my paper, I stated clearly that all the reactions I used were characteristic of aldehydes and ketones in general, and that none of them was specific for the adrenal cortical sterones. Hence, Dr. Gomori and I have no disagreement with respect to the chemical significance of the phenylhydrazine reaction.

Second, with respect to the Feulgen plasmal reaction, it is well known that the recolorization of leucofuchsin, or Schiff's reagent, is by no means specific for aldehydes. Lison has shown that Schiff's reagent can be recolorized by ketones, aldehydes, and other reducing groups. Hence the reaction Dr. Gomori used can be produced by ketones as well as by aldehydes.

Third, I was not aware that oxidation of tissues prior to phenylhydrazine treatment had any role in bringing out the reaction. Iodine, molecular oxygen in alkaline buffer, and indophenol were all used in my hands with a view toward eliminating ascorbic acid, which might give a false positive with phenylhydrazine. Dr. Gomori has pointed out that iodine or molecular oxygen might be responsible for bringing out the so-called "plasmal reaction." He made no mention of indophenol, although I reported that the phenylhydrazine reaction occurred after the tissues had been treated by indophenol. I should like to ask Dr. Gomori if he knows of any evidence that indophenol can bring out the plasmal reaction.

[Dr. Gomori made remarks to the effect that he knew of no such evidence.]

Fourth, with respect to the "plasmal reaction," Feulgen and Verne and their coworkers presented no conclusive evidence that the compounds they were dealing with were aldehydes and not ketones, as the reagents they used—Schiff's reagent, phenylhydrazine, semicarbazide, and thiosemicarbazide—are reagents for ketones as well as for aldehydes. The "plasmal reaction" can, hence, be produced by aliphatic ketones as well as by aliphatic aldehydes, and in this connection it is worth mentioning that the corticosterones are all aliphatic ketones.

Fifth, in spite of the fact that the phenylhydrazine reaction is not specific for the ketosteroids, but shows the presence of aldehydes and ketones of whatever nature, it is still, in my opinion, of localizing value in tissues under some circumstances. For instance, in the adrenal cortex of the cat, the

reaction is not present throughout the width of the cortex, but is present in a narrow band corresponding to the zone of spongiocytes. Cortical cells peripheral to this zone show no detectable yellow and, in the inner zones, the reaction is likewise absent or, in some cases, very weak. Hence, within the limits of sensitivity of this method, acetone-soluble, water-insoluble aldehydes and ketones of any sort cannot be detected in those cortical areas where the reaction is absent. Thus, it follows that the cortical sterones can be regarded as confined to and localized in some or all of those areas where the reaction is positive and absent elsewhere in detectable amounts. In the human, dog, and rat, the phenylhydrazine reaction occurs throughout the width of the cortex. In such cases, the reaction does not indicate any differential localization of ketones and aldehydes within the various zones of the cortex. Likewise, in the testis, Pollock showed the phenylhydrazine reaction to be positive in the interstitial cells and absent from the tubules. It follows that acetone-soluble, water-insoluble compounds with the carbonyl group (such as testosterone) are confined to the interstitial cells, which, hence, appear as a site of possible localization of any ketosteroids (such as testorenone) which may be in the testis, whereas the tubules do not give evidence of being such a site of localization.

Now, perhaps, the area of disagreement between Dr. Gomori and myself is not as great as it may have seemed originally.

# ENZYME SYSTEMS OF ISOLATED CELL NUCLEI

By Alexander L. Dounce

*School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.*

## *Introduction*

The preparation of nuclei on a large scale by chemical or physicochemical methods probably dates back to the work of Miescher, who isolated pus cell nuclei by a rather drastic method which included the use of a pepsin-hydrochloric acid mixture to digest away the cytoplasm. Miescher and Kossel also obtained nuclei from fish spermatozoa which were prepared by a rather mild procedure which would have permitted a subsequent study of enzymes in the isolated nuclei. However, at the time this work was accomplished, enzyme chemistry had not been developed to an appreciable extent. Much useful work might be carried out on enzyme studies of fish spermatozoa by any one having an available source of material.

Subsequent workers prepared bird erythrocyte nuclei by methods which would have permitted enzyme studies, but such studies were not made. For example, preparations of bird erythrocyte nuclei liberated by freezing and thawing were made by Warburg. References to this early work and to much of the later work can be found in a review article on gene structure and action by A. Gulick.<sup>1</sup>

## *Recent Methods for Preparing Isolated Cell Nuclei*

In more recent times, nuclei have been made from mammalian spermatozoa by means of sonic disintegration of the cells. The nucleated halves of sea-urchin eggs were prepared by Harvey, using a centrifugation technique. Cytochrome oxidase was found in the nuclei in both cases. These results have been discussed briefly by Dounce.<sup>2</sup>

Bird erythrocyte nuclei have been prepared by Yakushizi,<sup>3</sup> Laskowski,<sup>4, 5</sup> and Lan and Dounce.<sup>6</sup> The latter authors used the same method previously employed by Yakushizi,<sup>3</sup> but failed to realize it because they had not obtained the original paper, which was published in a Korean journal. We should like to acknowledge this oversight at the present time. Yakushizi also worked with pus cell nuclei.<sup>7</sup>

Although the bird erythrocyte nuclei obtainable by the most recent methods are admirably suited for enzyme studies, as far as mildness of the method of preparation is concerned, very little work of this nature has been carried out. Dounce and Seibel found acid phosphatase present in chicken erythrocyte nuclei.<sup>8</sup> The rate of oxygen consumption and anaerobic glycolysis of chicken erythrocyte nuclei has been studied by Hunter and Baufield.<sup>9</sup>

In the case of tissues of mammalian origin, two methods have been published in detail which have been used to obtain nuclei suitable for enzymatic study.<sup>7</sup> One of the methods is that of Behrens.<sup>10-15</sup> His procedure consists of drying the tissue in question in the frozen state, then cutting it up and pulverizing it in a ball mill. The pulverized material is sifted, repulverized,

and, finally, the nuclei are obtained by repeated centrifugation from a mixture of benzene and carbon tetrachloride. Behrens found little lipase in nuclei prepared in this manner, but considerable arginase.

The method of Behrens is, according to the numerous publications of the author, quite general and has been applied to plant tissue as well as to a number of animal tissues. It has been employed in a modified form by R. Williams *et al.*<sup>16</sup> to compare the vitamin content of nuclei from normal tissue and tumor tissue. The results obtained in this study are shown in TABLE 1. Since the assays were microbiological for the most part, it is difficult to state whether the vitamins were measured as such, or as parts of co-enzymes. The method of Behrens, in a slightly modified form, also has been used by Mayer and Gulick<sup>17</sup> to study the protein composition of nuclei.

It seems probable that the method of Behrens is the best procedure available at the present time for preparing in quantity nuclei upon which studies

TABLE 1  
VITAMINS IN NUCLEI  
(Micrograms of Vitamin Per Gram Dry Material)

<i>Vitamin</i>	<i>Whole beef heart</i>	<i>Beef heart nuclei</i>	<i>Whole mouse cancer</i>	<i>Mouse cancer nuclei</i>
Riboflavin	34	130	8.3	7.0
Inositol	7600	2000	450	400
Nicotinic Acid	320	900	130	95
Panthothenic Acid	75	270	60	43
Thiamin	32	90	9.0	7.4
Pyridoxin	4.4	4.2	0.87	0.90
Folic Acid	1.1	3.9	17	13
Biotin	0.52	0.25	0.35	0.27

are to be made of water-soluble constituents such as coenzymes and low molecular weight metabolites, because the use of an aqueous medium is altogether avoided. Since no photographs have yet been published of nuclei obtained in this way, the state of purity is not clear, although analyses for desoxyribonucleic acid indicate a reasonable degree of purity. The method is, however, laborious, time-consuming, and not well suited for preparing nuclei for studies of delicate enzymes which might be destroyed or considerably damaged by the procedures involved.

The second method described in detail which has been used to prepare on a large scale nuclei suitable for enzyme studies is the method of Dounce.<sup>2</sup> This method is based upon the earlier citric acid methods employed by Stoneburg,<sup>18</sup> Marshak,<sup>19, 20</sup> and Haven and Levy,<sup>21</sup> but far less citric acid is employed. The pH is maintained at approximately 6.0, where nearly all enzymes are perfectly stable, particularly in the cold. This method has been used by Lan<sup>22, 23</sup> in this laboratory to study enzymes of isolated liver-cell nuclei. Lipid studies have also been made on nuclei prepared in this way by Dounce<sup>24</sup> and by Williams *et al.*<sup>25</sup> Dounce<sup>26</sup> has determined desoxyribonucleic acid in nuclei prepared in the same manner. Hoerr has used

the method to obtain nuclei from liver cells.<sup>27</sup> The method has been used in a slightly modified form by von Euler and co-workers<sup>28-32</sup> to study nuclear enzymatic activity, but, more particularly, to study nucleic acid and nucleoproteins of the nuclei.

It is our opinion that, for gross analytical purposes where it is not necessary to maintain enzymatic activity, it may be best to prepare cell nuclei at pH 3.8 to 4 whenever possible.<sup>24, 26</sup> Although many enzymes may be denatured at this pH, there should be maximal retention of lipid, nucleic acid, and histone, and protein also should be quite well retained.

Since nuclei prepared at pH 4 or at pH 6, as described, may lose low molecular weight, water-soluble components such as coenzymes and substrates, if these materials were present when the nucleus was in the intact cell, material of this sort must be added when necessary in investigating enzyme systems of the isolated nuclei. Thus, the method of Dounce may not be suitable for preparing nuclei in which coenzymes, vitamins, or low molecular weight, water-soluble substrates are to be studied, and should not be used for studies of this kind, unless it can be proved that such constituents are not extracted from the nuclei during their isolation.

This statement applies to any method for preparing nuclei in which an aqueous medium is employed, with or without the addition of acid. However, in enzyme studies the principal object, at least at the outset, is to preserve the apoenzymes in an undamaged state. This appears to be accomplished by the method of Dounce.

Recently, the method for preparing nuclei at pH 6.0 has been improved in this laboratory by reversing the first steps of the procedure and using temperatures close to zero throughout the preparation. The latter procedure has been made possible by the acquisition of a refrigerated centrifuge.

Other workers have reported the separation of cell nuclei at zero degrees centigrade without the use of any citric acid for lowering the pH<sup>33-35</sup>. However, since no photographs have been published which show enough nuclei to enable one to get an idea of the state of purity of the preparation, we cannot easily compare such preparations with our own at the present time. We have never been successful in preparing good samples of nuclei without the eventual use of acid to lower the pH to 6.0, and we have found that somewhat better results are obtained with citric acid than with acetic acid.

In the improved procedure, 200 grams of frozen liver, after being cut into small pieces, are mixed in a previously cooled Waring Blendor, with 350 ml. of ice water containing 50 grams of crushed ice, for 45 seconds to one minute before adding any acid. Then enough 0.1 molar citric acid is added dropwise to lower the pH to a value between 5.8 and 6.0. We have found that 8 to 9 cc. of 0.1 molar citric acid are generally sufficient for this purpose, which amounts to 80 or 90 per cent of the quantity of citric acid used in the original method. Possibly the preliminary mixing of the liver in the blender with ice water causes the liberation of a slight amount of acid.

The blender is then allowed to run for 15 minutes. Three 50-gram portions of ice are added from time to time to keep the temperature as low as possible. The material is then strained through cheesecloth and the nuclei

are isolated as previously described.<sup>2</sup> If possible, all operations should be carried out in a cold room so that the temperature remains between zero and 5 degrees C. throughout the preparation. It is possible and perhaps advantageous to use 50-gram portions of liver instead of 100-gram portions, and to halve the amounts of all other constituents added. In this case, the total time of blending is reduced to 8 minutes.

The Waring Blendor used in this work has been model 14, which uses two amperes of current. Recently, we have found that the new model 15 Waring Blendor, drawing three amperes, destroys nuclei from liver cells. This blendor can be used, however, in conjunction with a rheostat which reduces the operating voltage to about 95 volts.

The improved method appears to eliminate an objection raised by Dr. Claude in a private communication. He thought that possibly in the original method only acid-fixed nuclei were being isolated, that is, nuclei which had been subjected to a low pH when the first few pieces of liver were added to the cold citric acid in the blendor. We had found, however, that the pH rises so rapidly after the addition of small amounts of liver that such an assumption was improbable. Nevertheless, the fact remained that some of the isolated nuclei had been subjected to lower pH than others, and hence the improved method is of distinct advantage.

One might inquire why we are so particular about the pH employed during the preparation of isolated nuclei. The principal aim in this respect was to use a pH as close to 7.0 as possible. It was found that between pH 4 and 6 an agglutination of cytoplasmic granules occurred to such an extent that separation of nuclei from the agglutinated mass was very difficult or impossible. At pH 4, the granules became dispersed sufficiently so that nuclei could easily be isolated. However, this pH is too low for sensitive enzymes. At pH 5.8 to 6.0, the cytoplasmic granules again became dispersed and isolation of nuclei was possible. If the pH was raised to 6.5, however, the nuclei disappeared during the process of centrifugation, possibly because of the action of cytoplasmic enzymes. Although at the present time most of our operations are carried out at zero degrees C., the temperature in the Waring Blendor may rise to 5° or 6° C. during the last few minutes of blending, and it might be at this point that the nuclei are dissolved.

It has been found that the addition of salts or buffers prevents proper breakdown of the cells at pH 6.0, so that these materials must be avoided if our method is being employed. It was found, furthermore, that isotonic saline could remove protein from isolated liver-cell nuclei to a greater extent than distilled water, which apparently removes very little.<sup>3</sup>

The use of protein solutions to prevent unwanted swelling of nuclei with subsequent extraction of nucleoprotein at pH values above 7.5 has been studied by von Euler.<sup>29</sup> It is not yet known whether this procedure can be applied to the preparation of nuclei for enzyme work.

The yield of nuclei prepared from liver cells by our improved method described above is between two and three grams per 100 grams of liver. The method has been designed to obtain as pure nuclei as possible rather than to obtain all the nuclei present.

It is an interesting question as to what factors are responsible for the liberation of nuclei from the cells in the methods involving aqueous media. It is certain that strong citric acid has a pronounced action in disrupting the cell membrane<sup>36</sup> and perhaps, also, the cytoplasm. At pH 6.0, where very little citric acid is used, this effect is much lessened, but appears still to be operative, since, as has been stated, citric acid is slightly better than acetic acid in preparing nuclei at pH 6.0. Part of the effect of the acid may be a pH effect and part an anion effect, possibly due to a complexing of magnesium and calcium which are found in the cell wall.<sup>37</sup>

Dr. Mirsky has pointed out in the discussion that the shearing effect of the Waring Blendor is of great importance in preparing nuclei. This shearing effect can be increased to a point where the nuclei themselves are completely disrupted, simply by using much less material so that the blendor can operate at higher speed. The model 15 Waring Blendor, when used without a rheostat, apparently disrupts liver-cell nuclei because of excessive speed.

Another factor which may aid in breaking the cells and liberating nuclei is a very slight autolytic action. We ordinarily freeze the liver before using it by placing it in the unit of a refrigerator. In this manner, the freezing is not instantaneous. When livers were frozen quickly by cooling in a calcium chloride-ice bath or in an acetone-dry ice bath, the cells were much more resistant, and it became difficult to obtain nuclei by our procedure. It is also possible that slow freezing might tend to disrupt the cell by formation of ice crystals.

Schneider has reported the preparations of nuclei by using the Potter-Elvehjem ground glass homogenizer on small samples instead of using the Waring Blendor.<sup>34, 35</sup> We have been able to obtain quite clean preparations of liver-cell nuclei by use of small samples with a ground glass homogenizer. Our homogenizer, which is of somewhat different design from the one originally employed by Potter and Elvehjem, is shown in FIGURE 1. The homogenizer is run from a motor, and an ice-water bath is used for cooling. The length of time necessary to break the cells will depend upon the speed of the motor and can be found by trial and error. The frozen liver is cut up into fine pieces before being added to the homogenizer. The outside portion of the homogenizer is raised and lowered, the plunger being held at a fixed level, in order to make certain that all pieces of the liver get into the bottom and become ground. If the homogenizer is run too long, there is a tendency to produce "eviscerated" nuclei which have been deprived of their chromatin. Such nuclei are perhaps in reality only nuclear membranes.

It has been possible to apply our improved method of preparing nuclei to sheep, dog, and human kidney and to sheep pancreas, using some modifications which are necessary to remove fiber. This work will be described elsewhere. Thus far, we have not been successful with thymus or spleen. However, von Euler prepared nuclei from thymus and other tissues<sup>28, 29</sup> by a modification of our original method, which involves a short exposure of the nuclei to pH 4.0 at zero degrees C., followed by isolation of the liberated nuclei at higher pH values.

We have found that tumor tissue is likely to be much more resistant than liver or kidney, as far as breaking the cells is concerned. It has been impossible, thus far, to obtain very good nuclei from Hepatoma 31 or from Walker Carcinoma 256 without the use of strong citric acid. This difficulty with tumor tissue also has been noted by von Euler.<sup>29</sup> We did succeed however in making a fairly good preparation of nuclei from a mouse leukemia.

In concluding the discussion of methods of preparing isolated nuclei by

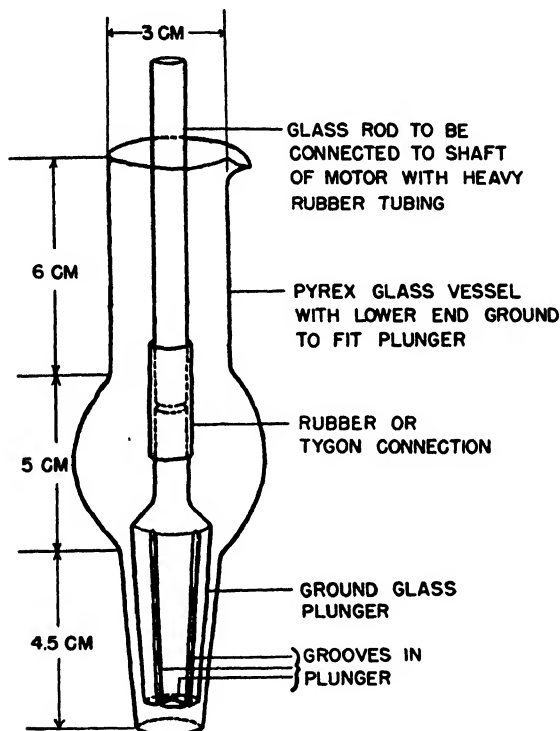


FIGURE 1. Ground glass homogenizer. Smaller models also can be used.

physicochemical methods, it should be stated that it is not enough to observe the liberation of some nuclei microscopically. It is necessary to break a very high proportion of the total cellular material present and subsequently to remove quite completely any remaining whole cells and, also, to remove the various cellular granules. It appears to us that articles on new methods of isolating cell nuclei should include photomicrographs in order that the reader may obtain some idea of the state of purity of the nuclei.

#### *Purpose of Studies of Isolated Cell Nuclei*

Before stating the results of enzyme studies of isolated cell nuclei, up to the present time, we should like to digress long enough to outline briefly



two hypotheses which are, at present, directing our general research aims and thinking. We are motivated by the hope of discovering something fundamental about cell division which can be utilized to throw light on the abnormal cellular growths which we call cancer. In understanding the phenomenon of cancer, a knowledge of the synthesis of protein and nucleic acid becomes important, as well as knowledge of sites where these syntheses occur.

It appears possible for nucleic acid and protein to be synthesized in the cytoplasm without cell division's taking place, as is evidenced by work on viruses and by the work of Spiegelman *et al.*, on the so-called plasma genes.<sup>38</sup> References will be found in Spiegelman's article to other work supporting this view. It is, furthermore, indirectly supported by the work of Brues, Tracy, and Cohn.<sup>39</sup> However, when cell division takes place in mammalian tissues, the nucleus participates and nuclear material as well as cytoplasmic material is synthesized. It is not implied that the nucleus never carries on metabolism unless the cell is dividing. Indeed, the contrary must be true at least in special cases, as evidenced by the work of Duryee reported in this monograph, and by indirect evidence such as that collected by Stern.<sup>40</sup> The interesting work of Brues, Tracy, and Cohn<sup>39</sup> on the uptake of radioactive phosphate by desoxyribo- and ribonucleoprotein indicates that, in liver cells, the nucleic metabolism is low in the nucleus in the resting cell and much higher in the dividing cell, whereas the nucleic acid metabolism in the cytoplasm, in either the resting or dividing cell, is considerable. Regenerating liver and tumor were used as sources of dividing cells, and nuclei were isolated by the use of strong citric acid in this work.

It occurred to us, after finding a number of enzymes present in the nucleus of the liver cell, that an important factor limiting metabolism in the nucleus of a resting cell might be the non-availability of certain important metabolites in the nucleus rather than a lack of enzymes. These metabolites might be low or absent because of their being screened out by the cytoplasm, after entering the cell. Such hypothetical screening could depend upon the presence of active enzyme systems in the cytoplasm capable of very rapidly transforming incoming metabolites into products not useful for the synthesis of protoplasm. For example, glucose might be rapidly turned into glycogen and deposited in an insoluble form, while amino acids might be rapidly deaminated and subsequently oxidized or converted to carbohydrate or ketone bodies; or they might be used up for protein synthesis in the cytoplasm.

According to this hypothesis, any factor which would permit metabolites necessary for protoplasmic synthesis to penetrate through the cytoplasm to the nucleus should favor cellular division. In the case of regenerating liver, the situation might be that of flooding the remaining liver with metabolites (because of the increased rate of blood flow) to such an extent that the capacity of the cytoplasm to assimilate the incoming material would be exceeded, with the resulting penetration of the metabolites to the nucleus. It seems possible that any factor directly or indirectly damaging cytoplasm might eventually reduce its metabolic capacity to the point where metab-

olites necessary for protoplasmic synthesis could penetrate to the nucleus. Such a cytoplasmic derangement, if permanent, would be classed as cancer. Other investigators<sup>41, 42</sup> have previously considered cancer as being primarily a disease of cytoplasm. It is even possible that an unfavorable mutation might indirectly cause cytoplasmic damage. Of course, obvious morphological nuclear derangements in cancer cells are well known, but it is possible that the cells which have nuclear derangements do not maintain themselves.

The cytoplasmic screening hypothesis just outlined is to be applied only to cells capable of division under the proper conditions. One piece of evidence which to some extent supports the hypothesis is as follows: It has been found by Chipps and Duff<sup>43</sup> that glycogen appears in the nuclei of liver cells in uncontrolled diabetes and in some other pathological conditions, whereas it is absent normally from the nucleus. We have found no glycogen in nuclei obtained from normal rat liver.<sup>24</sup> In diabetes, the excessive gluconeogenesis occurring in the liver, presumably in the cytoplasm of the cells, might well flood the cell with glucose to such an extent that this metabolite could penetrate to the nucleus and there become transformed to glycogen. We have recently found phosphorylase activity in nuclei isolated from rat-liver cells, and this finding appears to strengthen the above evidence.

It is possible that the hypothesis of cytoplasmic screening can be further investigated experimentally by comparing the concentrations of other substrates in isolated nuclei with their corresponding concentrations in cytoplasm or whole tissue. The method of Behrens is probably the only method available, at the present time, which is certain to leave water-soluble substrates in the nucleus, and which is therefore suitable for this purpose. For studying most substrates, microdissection probably would not yield enough material, although it might be very helpful in some cases.

A second general aim in studying isolated cell nuclei was developed as a result of Greenstein's conclusions<sup>44</sup> about the enzymatic pattern of malignant tumor cells. Greenstein has shown that tumors tend to exhibit a constant enzyme pattern. A given enzyme may be higher or lower in concentration in a tumor than in the tissue from which the tumor is derived, but the final enzyme pattern is reasonably similar for the various tumors studied. It is the rule, although there are exceptions, for cancer tissue to lose specialized metabolic function. That is, the cancer cell rarely serves the metabolism of the entire host, but instead serves only its own metabolism, which is the metabolism necessary for the synthesis of protoplasm.

It occurred to us that the main enzyme systems present in the nucleus might be those necessary for the synthesis of protoplasm, and that, in cancer, the enzyme pattern of the cytoplasm might revert to the enzyme pattern of the nucleus, since here, in both the nucleus and the cytoplasm, the chief function seems to be to synthesize protoplasm rather than to carry out specialized metabolic activities for the benefit of the organism as a whole.

If this idea is correct, the enzyme pattern of nuclei from ordinary resting cells should resemble the total enzyme pattern of cancer, which would be

similar in nucleus and cytoplasm. An investigation of this point is now under way, and in the following section it will be seen to what extent the enzyme pattern thus far obtained for isolated nuclei resembles the enzyme pattern of tumors.

These two hypotheses have been presented to show our general aims, and, also, to try to interest other workers in this field of research. Even if both hypotheses eventually should prove to be false, they may yet serve a useful purpose in promoting systematic work on cell nuclei.

TABLE 2  
ENZYMES IN ISOLATED RAT-LIVER CELL NUCLEI AND RAT HEPATOMAS

<i>Enzyme</i>	<i>Concentration in nucleus as per cent of concentration in whole tissue</i>	<i>Concentration in rat hepatoma 31 as per cent of concentration in normal tissue</i>
*Aldolase	40	—
†d-Amino oxidase	100	10
*Arginase	113	10
†Catalase	0.05–0.1	0.1
†Choline oxidase	0.0	0.0
Cytochrome oxidase	50–60	28
Cytochrome C	low	**22
*Enolase	50	—
Esterase	50	lowered
Alkaline phosphatase	192	13,550
Acid phosphatase	25–30 (minimal)	200
*Phosphorylase	66 after grinding 26 without grinding	—
*Lactic dehydrogenase	40	present
*Succinic dehydrogenase	0.0	††14
†Uricase	100 (minimal)	5

\* Nuclei prepared by the improved method.

† Work of T. H. Lan.

\*\* Primary tumor.

†† Mouse hepatoma.

‡ See footnote p. 991.

### *Results of Investigations on Enzyme Systems of Isolated Liver Cell Nuclei*

References to earlier work on the enzyme content of isolated cell nuclei are given in the paper of Dounce.<sup>2</sup> More recently, Dounce and Lan<sup>22–24</sup> investigated a number of enzymes in liver-cell nuclei isolated by the original method of Dounce.<sup>2</sup> The results of these investigations, with additional results on the enzymes aldolase, enolase; lactic dehydrogenase, and phosphorylase are shown in TABLE 2. The enzymes appear to fall into three classes. The first class comprises the enzymes arginase, d-amino oxidase, alkaline phosphatase, and uricase, whose concentrations appear to be as high or higher in the isolated nuclei than in whole liver. The second class comprises the majority of the enzymes investigated, whose concentrations were found to be roughly one half their concentrations in whole liver.

The most remarkable of the results thus far obtained, however, appears to us to be that the enzymes catalase, succinic dehydrogenase, and choline oxidase, which fall into a third class, are very low in concentrations in the nuclei as compared to their concentrations in the whole liver. Cytochrome

C was also low in concentration in the nuclei.\* Since the nucleus of the liver cell constitutes only about 8 per cent of the cell volume,<sup>20</sup> the concentration of an enzyme in whole tissue can be taken as roughly equal to its concentration in cytoplasm.

Succinic dehydrogenase is of particular interest because of its role in the carbohydrate oxidation cycle. In the case of succinic dehydrogenase, two techniques were employed for analysis, namely, the Thunberg technique, using the rate of decolorization of methylene blue in the presence of succinate and nuclei, and the Warburg technique for measuring oxygen consumption caused by the nuclei in the presence of succinate and added cytochrome C. The failure to detect appreciable succinic dehydrogenase by these methods might at first sight appear to conflict with the results recently obtained by Schneider.<sup>34</sup> However, Schneider's nuclei admittedly were contaminated with large granules from the cytoplasm, which contain a relatively high concentration of succinic dehydrogenase,<sup>34</sup> since Schneider's aim was to obtain all of the nuclei, rather than part of the nuclei in a highly purified state. Our nuclei do not appear to contain large granules in significant amounts. Great care has been taken to remove these by centrifuging the nuclei at low speed, and we find that Schneider's remark in the discussion following this paper about the difficulty of microscopic observation of these granules is unfounded as far as we are concerned.

Lately, using nuclei of rat-liver cells obtained by our improved method, we again have failed to demonstrate succinic dehydrogenase by the rate of decolorization of methylene blue in the Thunberg tube, and by the Warburg technique. Apparently then, the absence of appreciable succinic dehydrogenase can be used as one criterion of purity for samples of liver-cell nuclei prepared by mild procedures. In spite of the lack of succinic dehydrogenase activity, cytochrome oxidase was still readily detected, although a reinvestigation of this enzyme from the quantitative standpoint has not yet been made.\*

Of all the enzymes studied, alkaline phosphatase was found to be the only one appearing in considerably higher concentration in the nucleus than in the cytoplasm. This result seems to be in agreement with the results obtained by Willmer, using the Gomori technique, which show a high concentration of alkaline phosphatase in the chromosomes.<sup>45</sup>

Some results of enzyme studies of liver tumors made by Greenstein<sup>44</sup> also are shown in TABLE 2. It can be seen from a comparison of these results with the results of studies of the isolated nuclei that there is by no means complete agreement between the two in enzyme pattern. However, it is interesting to notice that the enzymes low in the nuclei, that is, catalase, succinic dehydrogenase, and choline oxidase, also are low in the tumors. Cytochrome C, likewise, appears to be low in the nuclei and in the tumors.

\* Since this article was written, a number of quantitative studies have been made of cytochrome oxidase, succinic dehydrogenase, and catalase in nuclei isolated from normal rat-liver cells by our improved method at pH 6.0. In agreement with previous work, succinic dehydrogenase was never found in detectable amounts in the nuclei, although cytochrome oxidase was always found and in concentrations approximating those previously reported. Our previous work with catalase was not confirmed, however, since this enzyme was found in liver- and kidney-cell nuclei in concentrations greater than 50 per cent of the concentrations typical of whole cells. This discrepancy is very likely due to lack of a refrigerated centrifuge when nuclei were first studied in this laboratory. We now have concluded that catalase is an important constituent of liver-cell nuclei. High catalase activities have also been found in kidney-cell nuclei.

It seems probable that too few enzymes have been studied thus far in isolated nuclei to determine for certain whether or not the enzyme pattern of the isolated nuclei resembles the common enzyme pattern of tumors. Furthermore, other kinds of nuclei must be studied before positive conclusions can be drawn.

The enzymes marked in TABLE 2 with an asterisk have been studied in nuclei prepared by our improved method, reference to which has been made previously. A detailed investigation of the enzyme aldolase has been published<sup>46</sup> and details of the work on the other enzymes will appear elsewhere.

In addition to the enzymes appearing in TABLE 2, it has been possible to demonstrate qualitatively the presence of xanthine dehydrogenase, malic dehydrogenase, and a triose phosphate dehydrogenase. Presumably, the triose phosphate dehydrogenase is 3-phosphoglyceraldehyde dehydrogenase, since it acts on synthetic dl-3-phosphoglyceraldehyde (very kindly supplied by Drs. H. O. L. Fischer and E. Baer) as well as the triose phosphate mixture produced by the action of aldolase on fructose diphosphate. Von Euler believes that thymonucleodepolymerase also is present in isolated nuclei,<sup>29, 30</sup> although his evidence does not appear to be conclusive.

Coenzyme I and flavine adenine dinucleotide both are detectable in nuclei prepared from liver cells by the method of Dounce,<sup>2</sup> but both are present in insufficient quantity fully to activate apoenzymes requiring them. For instance, coenzyme I must be added to obtain full activity of lactic dehydrogenase and malic dehydrogenase, and flavine adenine dinucleotide must be added to obtain full activity of d-amino oxidase.<sup>22</sup> In the case of coenzyme I, it is very possible that most of this material is extracted and lost during the washing of the nuclei with water. It is not so certain however that this happens with flavine adenine dinucleotide, since in this case the combination of the prosthetic group with the apoenzyme (at least for d-amino oxidase) appears to be more stable than in the case of coenzyme I.

It should be noticed that in the original determinations of lactic dehydrogenase in isolated nuclei carried out by Dounce<sup>2</sup> and, also, in the determinations carried out by von Euler,<sup>29</sup> no diaphorase was added, although the methylene blue technique was employed. This was unfortunate, since the limiting factor in the reaction could easily be a low diaphorase content of nuclei. Von Euler found a very low activity of lactic dehydrogenase in nuclei, but this method of preparation involved a short exposure of the nuclei at zero degrees C. to a pH of 4.0. This may have injured the apoenzyme or diaphorase or both. We have, however, recently found an apoenzyme concentration in the isolated nuclei of about 40 per cent that of whole tissue in experiments in which the system was completely activated with diaphorase. The activity is, thus, very marked and the decolorization of methylene blue takes place in five minutes or less using 0.1 ml. portions of suspended nuclei. Since the time of decolorization of methylene blue is much longer without the addition of diaphorase, the activity of the nuclei in respect to diaphorase is quite low. The very prolonged decolorization times recorded by von Euler must have been due to a lack of diaphorase or to the effect of the low pH which he employed.

A question of importance concerning the enzyme systems in cell nuclei appears to us to be whether the isolated nuclei can cause glycolysis. This question is now being investigated. Because of the probable removal of all intermediate substrates from the nuclei during their preparation (if indeed these substrates were there originally in appreciable concentrations), a long induction period would be expected before the formation of lactic acid could occur. Appreciable reaction velocity at each step is dependent upon the establishment of reasonable substrate concentration at each step. If one starts with fructose diphosphate, for example, the first two or three steps of glycolysis should be easily measurable in a time interval of a few hours, but subsequent steps probably would require many hours to become measurable unless very large quantities of nuclei were used. Moreover, if the total glycolytic system were to be measured, it would be necessary to add all coenzymes and metallic activators known to function in glycolysis, such as coenzyme I, adenosine diphosphate, magnesium, and possibly calcium and potassium.

Owing to the considerable length of time which probably would be required for the establishment of appreciable amounts of all intermediates in the glycolysis system, it does not, therefore, seem surprising that we have obtained no appreciable formation of lactic acid from the action of isolated liver-cell nuclei on fructose diphosphate, even in the presence of adenosine diphosphate, coenzyme I, magnesium, calcium, and potassium. Even if small amounts of lactic acid had been formed, however, its identification would have been difficult in the presence of larger amounts of earlier intermediates of the glycolysis system.

Because of the difficulties just mentioned, it is perhaps preferable to attack the problem of determining whether glycolysis can occur in the liver-cell nucleus by the more laborious method of determining the enzymes of the glycolysis system singly. Thus far, as has been indicated previously, we have found that aldolase, enolase, lactic dehydrogenase, phosphorylase, and triose phosphate dehydrogenase are present in the nuclei isolated from rat liver by our improved method. Aldolase has been investigated in detail by means of a new colorimetric method.<sup>46</sup>

#### *Discussion of the Validity of Enzyme Studies on Nuclei Isolated by Aqueous Extraction Procedures*

It has been argued, on the one hand by Barron,<sup>47</sup> that only positive results are of significance in studies of enzymes in nuclei isolated by the method of Dounce, and, on the other hand by Schneider (see discussion following this paper), that the enzymes cytochrome oxidase and succinic dehydrogenase appearing in his nuclei may well be caused by contamination of the nuclei by large granules. Dr. Claude, in the discussion following the presentation of this work, also argued that the enzymes found in the nuclei might have come from a contamination of the nuclei with large granules, since he has found many enzymes to appear in higher concentrations in the large granules than elsewhere.

The question of contamination of our nuclei with large cytoplasmic

granules has been dealt with, when it was stated that such contamination apparently did not occur to an appreciable extent. There is still another possibility for contamination, however, which is not so easily ruled out. This is the possibility that enzymes might be adsorbed from solution on the surfaces of the nuclei. One argument against this supposition, which already has been given,<sup>2</sup> is that the enzyme catalase, which ordinarily is very easy to adsorb and which has an isoelectric point near 6.0 (its isoelectric point is 5.7), is found only in very low concentrations in the nuclei. Another argument is that washing the nuclei in physiological saline extracts appreciable quantities of some enzymes and apparently causes the nuclei to shrink from loss of protein,<sup>2</sup> but the same enzymes are still found in the washed nuclei. It seems likely that enzymes adsorbed on the surface of the nuclei would be quite easily removed by this procedure. As a matter of fact, a small amount of hemoglobin which apparently is adsorbed in the nuclei is quite well removed by this procedure.<sup>2</sup>

We have recently completed experiments with arginase which indicate that this enzyme is not appreciably adsorbed by isolated nuclei.<sup>48</sup> Also, we have found that the phosphorylase activity of isolated rat liver-cell nuclei is markedly enhanced if the nuclei are broken by grinding in a ground glass homogenizer. If the phosphorylase were simply adsorbed on the nuclear surfaces, no such grinding should be necessary in determining its activity. Grinding is necessary, presumably, because the glycogen used as substrate has such a high molecular weight that it penetrates the isolated nuclei quite slowly.

Finally, such a variety of enzymes have been found in the nuclei in concentrations comparable to their concentrations in cytoplasm that it seems hard to account for so much enzymatic material if it is merely adsorbed on the nuclear surfaces.

It would be difficult to deal with the question of the washing out of enzymes from the nuclei during their preparation were it not for the fact that many enzymes have been found in the nuclei in reasonably high concentrations. It appears unlikely that the enzyme proteins are easily removed from the nuclei by washing with water. Returning to the case of catalase, it appears unlikely that this enzyme should be very completely washed out, since it has a molecular weight of 250,000 and is a globulin, while aldolase, for example, which probably has a much lower molecular weight,<sup>49</sup> remains in quite high concentration. The experiments with isotonic saline also point to the difficulty of removing enzymes from the nuclei by washing.

It has been argued by Dr. Claude in this monograph that it is more important to determine the total amount of enzyme present in various cellular fractions than to determine the concentration of enzyme in a particular fraction. Schneider has stressed the importance of accounting for all of a particular enzyme present in the cell by summing the amounts found in the various fractions.<sup>34</sup>

We have approached the problem from exactly the opposite point of view. Our intention has been to obtain nuclei in as pure a state as possible

and then to determine the enzyme concentration in the isolated nuclei. In the liver cell, as has been stated, the nucleus comprises only about 8 per cent of the cell volume.<sup>20</sup> Therefore, one could not expect to find more than 8 to 10 per cent of the total amount of a given cellular enzyme in the nucleus, assuming equal concentrations of enzyme in the nucleus and the cytoplasm. To find a quantity of enzyme in the nucleus equal to the quantity in the cytoplasm, the concentration of enzyme in the nucleus would have to be many times greater than the concentration in cytoplasm. The argument has been offered that it might be best to ignore the presence of an enzyme in a cell fraction unless the major amount of the enzyme in the cell is carried in this fraction. If this conclusion were applied to non-enzymatic constituents, we would be forced to state that the nucleus contained no protein, other than histone, no phospholipid or lipid, and no ribonucleic acid in the nucleolus. The only constituents remaining would be desoxyribonucleic acid and histone, which are not known to occur in appreciable amounts in the cytoplasm. It appears to us that to draw such a conclusion would be as erroneous as to argue that the nucleus itself should be ignored since it comprises such a small fraction of the volume of the cell.

In a very interesting paper by A. M. Clark,<sup>50</sup> it is demonstrated that digestion of food by *Amoeba proteus* is dependent upon the presence of the nucleus, and the suggestion is made that the digestive enzymes are synthesized as zymogens in the nucleus and become activated only after passing out into the cytoplasm. In addition, the statement is made that a peroxidase which occurs in the nucleus and cytoplasm of the amoeba, and which tends to accumulate at the surface of food vacuoles, also is dependent upon the nucleus for its existence, since in enucleated amoebas it does not appear in the cytoplasm following the ingestion of food.

The work of Duryee which is outlined in this monograph also indicates that at least in special cases the nucleus must carry out some sort of metabolism in the resting stage. Furthermore, it appears to be generally true that just before cell division some metabolism must occur in the nucleus to account for the thickening of the chromosomes and changes in staining properties which can be observed. Other evidence to support this statement will be found in Clark<sup>50</sup> and in the paper of Stern already mentioned.<sup>40</sup> Metabolism of any sort thus far known requires the presence of enzymes. Thus it appears logical that enzymes should occur in the nucleus.

#### Concluding Remarks

In this paper, we have tried to present a general picture of research concerned with enzymes in the cell nucleus and to present a point of view based on the relatively meager amount of information thus far available, which, it is hoped, will encourage other workers, including enzymologists, to enter this field of research. It appears that this will be necessary if results are to be obtained in a reasonably short time. There is a need for critical experiments designed to check the validity of work already accomplished.



It appears to us that, in selected cases, it might be possible to obtain sufficient nuclei by microdissection to permit enzyme determinations to be carried out. For instance, the Cartesian diver technique might be used with certain respiratory enzymes. The use of microdissection should provide nuclei in a condition very closely resembling their state in the whole cell, and would, therefore, be invaluable in checking the results obtained from nuclei prepared by large-scale methods. Microdissection already has been used to obtain nuclei in which the enzyme catalase was studied. This work, reported by Bundling,<sup>61</sup> shows that catalase is low in the nucleus of salivary gland cells of *Chironomus*, compared to its concentration in cytoplasm. Microdissection studies might also be useful in solving the problem of the state of nucleic acid in isolated nuclei, which has been outlined briefly by Dounce.<sup>24</sup> The use of histochemical enzyme tests will, also, be most appropriate in checking results, if a sufficiently wide range of tests can be developed.

We are of the opinion that work already done shows that many enzymes can be found in a cell nucleus in significant concentrations, and that now the enzyme patterns of nuclei, isolated from as many tissues as possible, must be determined. Before this task is accomplished, however, much work remains to be done in establishing various satisfactory methods for obtaining nuclei suitable for enzyme work from tissue other than liver, kidney, or pancreas, or in finding a method free from obvious drawbacks which can be applied to all tissues.

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### *Discussion of the Paper*

DR. WALTER C. SCHNEIDER (*Rockefeller Institute for Medical Research, New York, N. Y.*): In studies of the enzyme distribution in tissue fractions, two factors must always be considered: (1) the specific enzyme activity of the fractions and of the whole tissue and (2) the proportion of the total enzyme activity present in the whole tissue which is associated with each tissue fraction.

Dr. Dounce's results with cytochrome oxidase indicate that isolated rat liver nuclei had a specific activity which was 50 to 60 per cent as great as the specific activity of whole rat liver. In our own experiments (J. Biol. Chem. **165**: 585, 1946), rat liver cells were broken in water and separated by centrifugation into a nuclear fraction, a large granule or mitochondria fraction, and an unfractionated residue. The nuclear fraction contained all of the nuclei present in the original tissue as shown both by microscopic examination and chemical analyses for desoxyribose nucleic acid. The nuclear fraction was, however, contaminated by large granules and the nuclei had clumped in large masses. The following cytochrome oxidase values were obtained (the specific activities are expressed as cu. mm. O<sub>2</sub> consumed per mg. dry material per hour): rat liver, 197; nuclear fraction, 100; large granule fraction, 786; unfractionated residue, 37.8. The data confirm the results of Dr. Dounce, since the specific activity of the nuclear fraction was about 50 per cent as great as that of the original rat liver.

On the other hand, the data clearly show that the large granule fraction

was the only fraction in which the specific activity was higher than in the liver tissue. In view of the high specific activity of the large granule fraction and of the observation that the nuclear fraction was contaminated with large granules, it seems highly probable that the cytochrome oxidase activity observed to be associated with the nuclear fraction was due to contamination by large granules. This is even more clearly indicated by the data on the proportion of the total activity present in the rat liver which was found in each of the fractions: nuclear fraction, 5.4 per cent; large granule fraction, 74 per cent; unfractionated residue, 14.6 per cent. Thus, although the specific activity of the nuclear fraction was about one half as great as the specific activity of the original rat liver, the total amount of enzyme activity associated with this fraction represented only 5.4 per cent of the total enzyme activity present in the rat liver.

It is impossible to tell whether the cytochrome oxidase activity which Dr. Dounce observed to be associated with isolated rat liver nuclei could be explained in terms of contamination with large granules. In this connection, it might be mentioned that large granules in hypotonic solutions are very poorly defined in the light microscope and that their presence might easily be overlooked unless special precautions were taken. Dr. Dounce has also found that succinic dehydrogenase was present only in traces in isolated rat liver nuclei. Our own results indicated that the ratio of succinic dehydrogenase activity to cytochrome oxidase activity was essentially the same in the liver fractions as in the whole liver. Since the succinic dehydrogenase activity of rat liver is only about one-third as great as the cytochrome oxidase activity, the possibility that insufficient amounts of nuclei were used by Dr. Dounce to detect succinic dehydrogenase activity must be considered.

It should also be pointed out that, in our own experiments, over 90 per cent of the total activity of each of three enzymes studied, present in the original liver tissue, was recovered in the tissue fractions. Thus, there was no possibility that unknown co-factors necessary for the enzyme assays and present in the whole liver tissue were lost in the preparation of the tissue fractions. The need for determining the specific activity and the total activity of the enzyme in the whole tissue and in each of the tissue fractions cannot be emphasized too strongly, for only in this manner is it possible to demonstrate that the techniques of enzyme assay are valid and to assess the significance of the specific activity of an enzyme in a given tissue fraction.

DR. A. L. DOUNCE: As has been emphasized in the paper, we believe that, if one wishes to study the composition of nuclei, the first step should be to obtain the nuclei in as pure condition as possible. Recovery of all nuclei present, or of 90 per cent of a constituent present in the cell, appears somewhat irrelevant in view of the relatively small percentage volume occupied by the nucleus in the case of the liver cell. We believe that other methods, such as microdissection and histochemical techniques, should be employed to check results obtained in large-scale experiments on isolated nuclei.

DR. JEAN BRACHET (*Department of Zoology, University of Pennsylvania, Philadelphia, Pa.*): Dr. Dounce rightly emphasized the need to compare

the enzymatic constitution of nuclei isolated by the method he used and by microdissection. A few years ago, I made a study of some of the enzymes present in the isolated germinal vesicle of the frog oocyte as compared with the cytoplasm. No respiratory enzymes have been studied so far, but dipeptidase, esterase, alkaline phosphatase, ribonuclease, and arginase were all found to be present in concentrations lower in the nucleus than in the cytoplasm.

It is noteworthy that, according to Duspiva, there is a loss of dipeptidase from the nucleus into the surrounding physiological saline solution which starts as soon as the germinal vesicle has been isolated. This finding, indicating possible loss of enzymes from the nuclei after their isolation, should be kept in mind. As regards the low content of alkaline phosphatase of the germinal vesicle compared to the high concentration of the same enzyme in other nuclei, as found by Dr. Dounce and many other workers using Dr. Gomori's technique, it may be said that the germinal vesicle is very poor in chromatin and rich in nuclear sap, and we know, of course, that the Gomori test is strongly positive in the chromatin and in the nucleoli only.

DR. A. L. DOUNCE: It is much to be hoped that the work mentioned by Dr. Brachet will shortly be published with details as to methods employed and results obtained on the percentage concentrations of the enzymes in the nuclei as compared with their concentrations in cytoplasm.

We have found that physiological saline tends to extract enzymes from isolated nuclei to a considerably greater extent than does distilled water, as mentioned in the paper.

# THE FUNCTION OF CELL INCLUSIONS IN THE METABOLISM OF *CHAOS CHAOS*

By Heinz Holter

*Cytochemical Department, Carlsberg Laboratorium. 8, Gl. Carlsbergvej.  
Copenhagen, Valby. Denmark.*

The starting point for the present studies consisted of a series of observations on the fate of the visible cytoplasmic inclusion bodies in the giant ameba, *Chaos chaos*. These inclusion bodies, shown in FIGURE 1, are: the crystals, the heavy spherical or refractive bodies, the fat droplets, and the food vacuoles.

Of the four inclusion bodies under consideration, two are enclosed within vacuoles, namely, the food balls and the crystals. The physiological function of the food balls is obvious. Regarding the other three, however, opinions have differed, but due to the work of Mast and his co-workers,<sup>1</sup> they are most commonly regarded as reserve food. According to this hypothesis, the contents of the food vacuoles are transformed within the vacuoles into crystals, *etc.* Then the food vacuoles divide into smaller vacuoles, thus spreading their contents over the whole of the ameba's cytoplasm. The non-vacuolized, refractive bodies and the fat droplets disintegrate and leave the food vacuoles by diffusion to be regenerated later in the cytoplasm. The food reserve, so created and distributed, is to be consumed during starvation.

Andresen and the author were interested in the starvation metabolism of *Chaos chaos* and, in order to study the role of the various microscopically visible inclusion bodies, we repeatedly counted their numbers in starving amebas<sup>2</sup> and determined at the same time the decrease in the ameba's volume induced by starvation.<sup>3</sup> Details concerning the technique of these measurements need not be discussed. The result of one typical experiment is shown in FIGURE 2. It will be noted that the concentration of the crystals observed in this instance did not decrease, but actually increased during starvation.

By taking into account the simultaneous change in the total volume of the starving amebas and the total number of crystals in the cytoplasm, rather than their concentration in a microscopic field, one finds that this number remained approximately constant (dotted lines in FIGURE 2). Similar results were obtained with the heavy spherical or refractive bodies.

Regarding the fat droplets, however, the individual variations between single amebas were so great and the results of our counts so erratic that we were unable to draw any unambiguous conclusion except that they did not disappear consistently and gradually during starvation. Concerning the crystals and heavy spherical bodies, on the other hand, one may feel justified in doubting the assumption that they constitute the ameba's reserve food, since it is obvious that they are not utilized during starvation to any appreciable extent.

Before an alternative hypothesis is offered, a few other observations should be mentioned. One concerns the fate of the food vacuoles. If large num-

bers of paramecia are fed to amebas which were slightly starved and therefore did not contain food remnants, the amebas produce many easily recognizable food vacuoles. The total number of such vacuoles formed within each ameba may be counted. Within a few hours after ingestion, the paramecia have been transformed into compact spherical balls. During the next day or two, these vacuoles become smaller and still more compact without ever being broken up and distributed into vacuoles which could compare with the crystal vacuoles in size or number. During the first 48

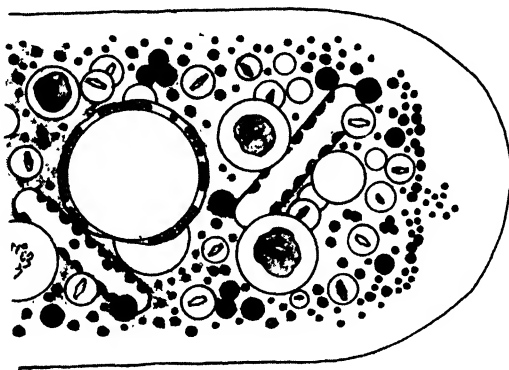


FIGURE 1. Cellular inclusions in *Chaos chaos*. Tip of pseudopodium showing two rod-shaped nuclei, one large contractile vacuole (coated with mitochondria), four food vacuoles, and numerous crystal-containing vacuoles. Refractive bodies (empty circles) and fat droplets (solid circles) are equal in size to the crystal-containing vacuoles. In addition, there are numerous smaller cytoplasmic inclusions.

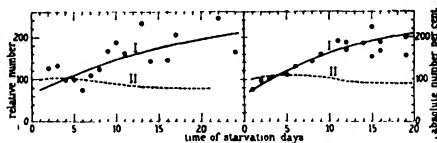


FIGURE 2. Number of crystals in relation to starvation. Curve I. Number of crystals (longer than 1 micron) per counting volume. Curve II. Total number of crystals in entire ameba. Data based on two independent series.

hours, at 22°C, after ingestion, practically all these food balls are defecated and, since they remain recognizable for some time after defecation, one is able to determine that the number of egested balls corresponds to the number of food vacuoles formed.

Another observation concerns the phenomenon of vacuole coalescence,<sup>2, 4</sup> which consists of a fusion of various types of permanent vacuoles other than the contractile vacuoles. This fusion is preceded by a period of agglutination that leads to a mixture of the vacuolar contents (FIGURES 3 and 4). Similar processes may also include the refractive bodies which, in this respect, behave like vacuoles.

The details of all observations cannot be reported here. The collected evidence, however, suggested the following hypothesis: in the food vacuoles,

there is a progressive breakdown of the food balls and an absorption of the soluble degradation products through the vacuolar wall. Physiologically, the food vacuoles correspond to the intestinal tract of the metazoa, since

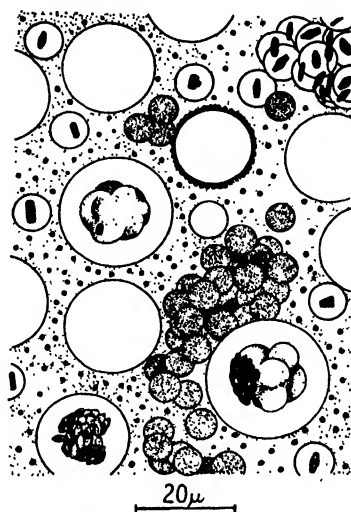


FIGURE 3. Cytoplasmic inclusions in 18-day starving amoeba, four days before death. A group of agglutinated crystal vacuoles is shown in upper right corner. Below them, and to the left, is shown a contractile vacuole coated with mitochondria. At its side are three agglutinated heavy spherical bodies. Below these is shown a vacuole with several coalesced spherical bodies and two crystals. Another similar vacuole, but with a cap of crystals on the spherical bodies, is shown at the lower right corner. The moruloid mass to the left represents a group of partially agglutinated, heavy spherical bodies. At the lower left corner is a vacuole containing a concretion of crystals. In addition, the cytoplasm contained numerous empty vacuoles, uncoated by mitochondria.

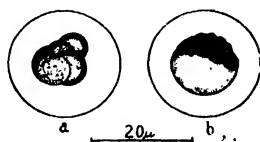


FIGURE 4. Typical coalescence vacuoles: (a) an early coalescence vacuole containing five heavy, spherical bodies; (b) an older coalescence vacuole with numerous crystals forming a cap on a structureless body which may have developed from the coalescence of heavy, spherical bodies.

the vacuolar wall constitutes the inner surface of the amoeba and the food substances do not, strictly speaking, enter the cytoplasm of the amoeba unless they diffuse through the vacuolar membrane.

The formation of crystals, heavy spherical bodies, and fat droplets is

assumed to occur in the cytoplasm, and, if any of these cytoplasmic inclusions were observed in the true food vacuoles, they probably were formed by vacuole coalescence and not by the primary transformation of food balls. In some respects, the coalescence of vacuoles gives the impression of being one step in a sequence of processes leading to the expulsion of formed excretory products. With crystals, the first step in this process would be the formation of a vacuolar membrane around the crystalline particles. As soon as this is formed, the contents of the vacuoles are already outside the cytoplasm. The next step is the collection of larger lumps of refuse by vacuole coalescence. The final step is egestion (FIGURE 5).

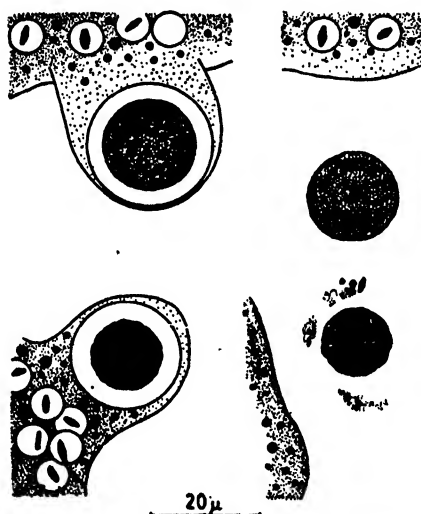


FIGURE 5. Two examples of egestion. Upper group: Egestion of food vacuole. Lower group: Egestion of a druse of crystals. The left figures show the bodies in the tip of a short pseudopodium of hyaline cytoplasm. Mitochondria, fat globules, and crystal vacuoles are present at the base of the pseudopodium. The right figures show the expelled objects lying free in the medium. Note small amount of extruded cytoplasm surrounding the crystal druse.

The defecation of crystal druses formed by the coalescence of crystal vacuoles has been frequently observed.

In the starving amoeba, the coalescence phenomenon seems to get out of hand, and huge concretions of crystals, which one may observe during the later stages of starvation, are not defecated. These must, therefore, be regarded as pathological.

The results of the counting experiments indicate rather strongly, in our opinion, that whatever the physiological function of the crystals and refractive bodies may be, it is hardly that of reserve food.

Zeuthen and the author,<sup>12</sup> therefore, attempted to study the starvation metabolism of these amoebas by suitable chemical means. A very promising method, based on the Cartesian diver technique, was developed by Zeuthen.<sup>6</sup>



It is the application of the diver principle to the weighing of small objects under water. The "diver balance," as Zeuthen calls it, is shown in FIGURE 6.

Regarding the principles and previous applications of Linderstrøm-Lang's Cartesian diver,<sup>6</sup> reference must be made to the literature.<sup>7</sup> Only the diver balance can be described. It consists of a small glass flask with a very long capillary tail. The wall and bore of the latter are much thinner than shown in FIGURE 6. The body of the flask contains an air bubble which makes it float in an inverted position. The capillary tail permits a rapid adjustment of the gas volume according to the applied pressure while, at the same time, minimizing the loss of gas by diffusion into the surrounding medium. At the top of the inverted flask is placed a cup of polystyrene, which, because of its low density, does not make the diver top-heavy. The

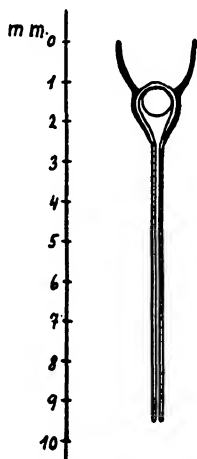


FIGURE 6. Diagram of the Cartesian diver balance. Note the cup of polystyrene mounted on the inverted capillary flask.

diver balance floats in a suitable medium consisting of water or balanced salt solutions, and its equilibrium pressure can be determined with an accuracy of about one or two mm. of water.

Weighing is performed by determining the equilibrium pressure of the "empty balance," then pipetting into the cup the object to be weighed and determining the equilibrium pressure of the "loaded balance." The resulting difference is the so-called "reduced weight" of the object, that is to say, its weight minus the weight of the volume of water displaced. These values alone are very useful reference quantities in biological work, as they provide a measure of the non-aqueous components of the cytoplasm investigated.

If necessary, the absolute weight can be readily determined. This is done by measuring the reduced weight in two media of different densities, one of them being, for instance, a starch solution, the other, water. From the two reduced weights, the absolute weight, the specific gravity, and, accordingly, the volume of the object can be calculated.

In its present dimensions, the diver balance permits the determination of reduced weights in the order of a few gamma with an accuracy of  $\pm 2$  per cent. The chief asset of the weighing method is, of course, that it does not injure the object. In our experiments, the individual amebas could be weighed over and over again, and between weighings they could be used for other purposes, *e. g.*, for determining the oxygen consumption in the ordinary micro-respirometer diver.

FIGURE 7 shows the course of such an experiment for four individual amebas. The abscissa in all examples gives the time of starvation in days. The curves of the lower chart give the reduced weights in gammas. At the time intervals marked by dotted lines, the oxygen consumption of the same animals was measured in standard divers, and the middle chart gives

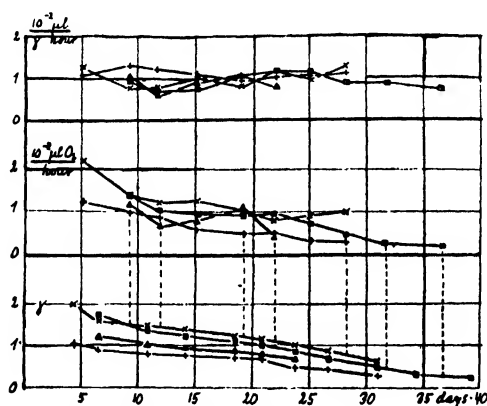


FIGURE 7. Changes in reduced weights and respiratory rates of four individual amebas during starvation. Upper curves represent oxygen consumption in microliters per hour per gamma of reduced weight; middle curves show oxygen consumption per hour; and lower curves give changes in reduced weights in gammas. Days of starvation given in abscissa.

the respiratory rates in micro-liters of oxygen consumed per hour. The upper chart is the ratio between the two sets of values. These ratios are practically constant throughout the experiment, thereby indicating that the respiratory rates depend roughly on the total amounts of cytoplasm, as was to be expected from previous experience.

How can such data elucidate the metabolism of starving amebas? It is obvious that the changes in reduced weight must result from the disappearance of substances with a specific gravity that differs from water. The following densities are assumed for the non-aqueous components of the ameba's cytoplasm:

Proteins	1.35
Carbohydrates	1.55
Fats and lipids	1.0
Salts	2.1

The values, of course, are based on rough estimates, but they give a general idea of the magnitudes involved. The bulk of the cell's substance undoubtedly consists of proteins. Regarding the lipid density, this is based

on the following values: 0.95 for fats, and ca. 1.03 for compound lipids. There are reasons to believe that the amounts of phospholipids and fats are essentially identical. The average density of lipids in the cell, therefore, must be close to 1.0.

With regard to salt content, we have assumed that the ameba regulates its total salt concentration to a constant value, at least as long as its condition can be regarded as non-pathological. This means that, during starvation, salts are gradually excreted in rough proportion to the amount of protein metabolized. If we make this assumption and if we further assume the total value of the normal osmotic concentration to be about equivalent to 0.025M NaCl,<sup>8</sup> the salt loss can be accounted for by adopting a slightly higher value for the specific gravity and reduced weight of the protein. Numerically, this works out to an increment of 0.02, thus bringing the specific gravity of the proteins to 1.37. Fortunately, this is a rather small correction. On the basis of these assumptions, the factor converting the observed change in reduced weight into absolute weights of protein is 3.7.

With regard to carbohydrates, the corresponding factor would be 2.9. The carbohydrate content of the ameba, however, is very small. For reasons to be explained later, it is possible to disregard completely the possible loss of carbohydrates during starvation. A loss of lipids cannot be measured, since their average density is the same as the water in which the diver balance is floating.

If these assumptions are accepted, the evaluation of the experiments becomes simple. We know the ameba's oxygen consumption and the amount of protein that has disappeared within a given interval of time. We know, from studies on other tissues and organisms, the amount of oxygen needed to combust the protein which has disappeared. If the total oxygen consumption is larger than these values (disregarding the carbohydrates), the difference must have been used for the combustion of substances which do not influence the reduced weight, in other words, for the combustion of lipids.

A close inspection of the curves in FIGURE 7 shows that the decrease in reduced weight has a tendency to slow up after the first few days of starvation. This tendency is brought out more clearly in FIGURE 8, where the decrease in reduced weight is plotted daily in per cent of the preceding volume.

In the middle of the starvation period, we may expect that the combustion of lipids will be highest compared with the combustion of heavier cytoplasmic components. Of the two examples considered in TABLE 1, Ameba No. 5 was selected for high lipid turnover. This ameba was characterized by a normal respiratory rate but rather small changes in reduced weight over a 10-day period. Ameba No. 11 was followed throughout the whole period of starvation, beginning at the time where the greatest part of the primary food vacuoles had disappeared.

The data in TABLE 1 show, in both instances, considerable differences between the actually observed oxygen consumption and the oxygen con-

sumption accounted for by the change in reduced weight, assuming, of course, that these changes were entirely due to the combustion of protein (corresponding oxygen consumption). These differences in oxygen consumption have been called lipid oxygen, and the corresponding amount of combusted lipids in absolute weight was, in Ameba No. 5, nearly the same as the total protein loss in the period considered. Even in Ameba No. 11, where the whole starvation period was included in the calculation, the lipids form a considerable portion of the total substance metabolized.

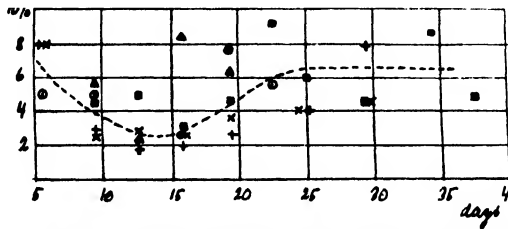


FIGURE 8. Percentage decrease in reduced weight during starvation. Decrease in reduced weight plotted daily in per cent of preceding volume.

TABLE 1

	Ameba no. 5		Ameba no. 11	
	Days of starvation			
	10 to 20		4 to 27	
$\Delta$ Reduced weight	0.14 $\gamma$		0.59 $\gamma$ *	
Corresponding protein	0.52 $\gamma$		2.18 $\gamma$ *	
Corresponding oxygen	0.52 $\lambda$		2.18 $\lambda$ †	
Observed $\Delta$ oxygen consumption	1.53 $\lambda$		3.90 $\lambda$ †	
Lipid oxygen	1.01 $\lambda$		1.72 $\lambda$ †	
Corresponding lipid	0.50 $\gamma$		0.86 $\gamma$ *	
* $\gamma$ Lipid/ $\gamma$ Protein	0.96		0.39	
cal. Lipid/cal. Protein	2.2		0.89	
$\Delta$ Reduced weight, 4th day	—		0.98 $\gamma$ *	
Absolute weight	—		50.0 $\gamma$ *	
Volume	—		0.049 $\lambda$ †	
Maximum protein	—		3.6 $\gamma$ (7.2%)*	
Minimum lipid	—		0.86 $\gamma$ (1.7%)*	

\* $\gamma$  = gamma =  $1 \times 10^{-6}$  gm.

† $\lambda$  = microliter =  $1 \times 10^{-6}$  liter.

This becomes still more evident if we compare not the absolute amounts of the substances but their energetic equivalents when converted roughly on the basis of factors known from human physiology. On this basis, Ameba No. 5 had, in the period considered, covered two-thirds of its energy requirements by combustion of lipids. In Ameba No. 11, nearly one-half of the total energy utilized during the entire starvation period had been supplied by lipids.

On the basis of the assumptions made, the values for reduced weight and oxygen consumption can also be used for a rough calculation of the initial

protein and lipid content of the ameba cytoplasm prior to starvation. For Ameba No. 11, these calculations gave a maximum value of 7.2 per cent for protein content and a minimum value of 1.7 per cent for lipid content (see TABLE 1).

The protein content thus determined cannot be checked by analysis of the same ameba, but a direct nitrogen determination of 15 amebas in the same state of nutrition, by a micro-Kjeldahl method,<sup>9</sup> gave 5.0 to 7.5 per cent protein, with a mean value of 6.4 per cent. The amount of lipid used by the ameba during the period of observation was 1.7 per cent of its initial wet weight. This, therefore, is the minimum amount of lipid present at the beginning of the experiment and, as pointed out before, it is determined on the assumption that the only other material oxidized is protein. If we substitute carbohydrate, with its higher density, as part of the fuel, the proportion of lipids used becomes still higher. This is why we did not consider the carbohydrates in our previous calculations.

The lipid value is only a minimum in any event, and the actual lipid content must be considerably higher. But even the minimum value is interesting in view of the fact that, in previous determinations of the contents of visible fat in the cytoplasm of *Chaos chaos*,<sup>2</sup> the highest value ever found was 0.5 per cent of the ameba's volume, while the average was much lower. This indicates that the microscopically visible fat droplets do not form the most important metabolic lipid resource of the amebas.

In other amebas that had been treated in the same way as the two examples described here, we have found varying proportions between lipids and heavy substrates combusted, but, in all instances so far analyzed, the invisible cytoplasmic lipids seem to constitute an important source of energy to the starving ameba. In the initial and final periods of starvation, heavy substrates usually dominate, but, in the middle periods, the lipids often play the main role.

In this connection, it should be noted that the period of excessive vacuole coalescence seems to set in when the period of slow decrease of the reduced weight is over, in other words, when the lipid content of the cytoplasm has become low. It is tempting to assume that the two phenomena are connected. It might mean, for instance, that the surface of the vacuolar membranes is dependent on the presence of a certain amount of cytoplasmic lipids.

What independent evidence can be produced to support the rather numerous assumptions made and the perhaps too far-reaching conclusions drawn? The possibility of checking the calculated protein content by direct Kjeldahl analysis has already been mentioned. Determinations of total lipid contents in amebas, according to the method of Schmidt-Nielsen,<sup>10</sup> should also be possible and will be attempted.

Respiratory quotients can be determined in the standard diver.<sup>11, 12</sup> This has been done in several instances, and the R. Q. values obtained so far have ranged between 0.75 and 0.88, which is consistent with the assumption of a mixed combustion of lipids and proteins. Here again, further work is needed.

So far, we have not been able to search the literature exhaustively for similar data on related material. But it ought to be mentioned that Parpart<sup>13</sup> and Hunter<sup>14</sup> found a total lipid content of 5.4 per cent wet weight in the cytoplasm of *Arbacia* eggs, and 77 per cent of the total lipids behaved as if they were cytoplasmatically bound. Heilbrunn<sup>15</sup> has reported on the release of bound lipid from the cytoplasm of *Arbacia* eggs and of *Ameba proteus* induced by the action of ammonium salts.

Finally, the author wishes to point out that the main object of the present paper was not the rather limited field of the metabolism of *Chaos chaos* but, rather, a demonstration of the general usefulness of Zeuthen's diver balance for cytochemical and cytophysiological research.

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## CONCLUDING REMARKS

By Robert Chambers

*Department of Biology, New York University, New York, N. Y.*

A general criticism which can be made of any experimental study for elucidating protoplasmic activities is the fragmentary nature of the conclusions to be drawn from the results achieved. We must always be aware of the necessarily limited view, but not lose hope that other aspects will eventually add with ours toward further revelation. Some of us, even most of us, may be off the main path, but, provided we painstakingly describe the methods and the precise nature of the material we use, we may be certain that at least some fraction of the results will have its value.

One of the pregnant problems of cellular physiology is the nature of the limiting boundary of the protoplasmic body of a cell. Our eyes should be kept open to the possibility of serious error in interpreting any limiting envelope after its removal from the internal cytoplasm.

We should also be on the alert in interpreting observations of isolated constituents of cells. The isolation involves destruction of the cell. In this process many changes occur, which not only affect the visible morphology, but the more subtle chemical and physical structure as well. The nucleus, as it is being isolated, most probably loses or gains materials from the dying cytoplasm. By the same token, products isolated from cells are exposed to cytolytic reactions. When separated, for example, by centrifugation, the formed elements may be different in any given respect from the granules visible in the living cell. Some of the segregated granules may have appeared during cytolysis; others may have lost or assumed properties which they did or did not have in the living cell.

The study of these cellular products has its own value. Their interpretation can be assisted by histochemical reactions within the integrated cell. Here again there is room for question, since most of the histochemical methods at our disposal are used on cells which have undergone the drastic changes of histological fixation. Some chemical observations have been made on structures *in situ* on the living cell. The conclusions drawn from results of the isolation experiments must be in accord with what can be obtained on the living cell.

Micromanipulative studies are of great value for an understanding of physical structure. Those who are experienced with the technique are astonished at the relative lack of damage when a microneedle is inserted into a living cell. Even here it is necessary to appreciate the resulting changes and to draw conclusions only when the changes can be shown to be reversible.

A study of model systems should be encouraged in attempts to reproduce structures which seem to be present in the living cell. An example of this, presented in one of the papers of this symposium, is the study of artificially produced protein fibers. This should be done irrespective of

whether the structures duplicate the more intimate ones in protoplasm. We have learned much from the model systems early developed by Butschli and Ludwig Rhumbler.

I conclude with a story told by Dr. Albert Szent-Gyorgy in one of his recent lectures on muscle contraction. A holy man asked to be given a glimpse of heaven. This plea was accorded him, and, when he looked in, lo! he found himself gazing at the portals of a second heaven. Let us look into our heavens, but we must expect that there will be other heavens still to be looked into.





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ANTI-HISTAMINE AGENTS IN ALLERGY\*

*Conference Chairman*

Fredrick F. Yonkman

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## INTRODUCTORY REMARKS

By Fredrick F. Yonkman

*Director of Research, Ciba Pharmaceutical Products, Inc., Summit, New Jersey, and Lecturer in Pharmacology and Therapeutics, College of Physicians and Surgeons, Columbia University, New York, N. Y.*

On behalf of the Organizing Committee, cooperating with Mrs. Eunice Thomas Miner, Executive Director of The New York Academy of Sciences, I should like to express my pleasure in introducing this monograph. Thanks are due to Dr. Dragstedt and the late Dr. SubbaRow for their generous and able assistance in the construction of the conference and especially to Mrs. Miner for attending so efficiently to the many details that are part and parcel of a conference of this scope.

As a Committee, we are most appreciative of the excellent suggestions regarding participants in the conference, both as to speakers and discussants, and we extend our genuine gratitude to the several anonymous supporters who made possible the assembling of our illustrious speakers, whose workshops and clinics are so widely separated geographically. Our chief regret is that numerous other renowned contributors to the scientific advances in this particular field could not have been invited to participate. There were reasonable limits which we could not exceed, especially those of time and support. Had both been adequate, however, certainly it would not have been difficult to have extended the conference for a longer period.

As one scans the Table of Contents of this monograph, one appreciates the historical developments concerning histamine, its pharmacodynamics, and its varying role in anaphylactic and allergic situations. We are aware of the devious methods whereby man has sought to dampen histamine's physiologic behavior. In one phase, it was degraded or split into its components; in another, it was coupled with cooperative partners. More recently, however, it is being given the "physiological brush-off" by handy whisk-brooms such as are now known, correctly or incorrectly, as "antihistaminic agents." What is next? If histamine is a chief offender in anaphylaxis or allergy, will someone some future day manage to dampen its activities by means other than those now at our disposal? This is highly probable, and one is almost sufficiently foolhardy to venture the prediction that such will be the case. What the next approach will be is problematic, but perhaps some clues may be found among the papers included in this monograph.

Recently, there has been some criticism of the term "antihistaminic" as employed for the various derivatives of the original Fourneau compounds. Much of this criticism seems warranted, since there is ample evidence that many of these substances exhibit other prominent pharmacodynamic actions. They are, to a varying degree, anticholinergics, local anesthetics, central sedatives, or oddly enough, in some instances, analeptics and even potentiators of certain physiologic responses. In fact, these new agents can manifest many actions simultaneously. Justly one asks why they are classified as antihistaminics. With equal justification, one might inquire as to how they might otherwise be classified. Since their chief pharmaco-

dynamic characteristic is that of nullifying the action of histamine, they are logically identified as antihistaminic agents, whereas, if one chooses to categorize them from a therapeutic point of view, they probably should be classified as antiallergic agents along with epinephrine, ephedrine, and similar compounds.

Although most of the more recently developed synthetic variations of the original Fournau and other compounds exhibit varying but marked capacities for nullifying the physiologic effects of histamine, their somewhat similar potency in terms of antianaphylactic action is not only fascinating but challenging. This variance in antihistaminic but not in antianaphylactic potency is, however, no more provocative of thought than is the finding of Abramson and coworkers in their experiments dealing with iontophoretic administration of histamine to the skin. By *reversed* iontophoresis, the histamine was detectable in the dermal fluid thus obtained. When ragweed pollen extract was administered in the same way, however, allergic whealing resulted, but then, after *reversed* iontophoresis, the dermal fluid contained no histamine or H-substances. Failure to detect histamine or H-substances in the fluid could reflect a true absence of these compounds, but it remains to be proved that they were not present because of possible linkage in a conjugated or nonreactive form, a condition which could militate against detection. Granted, however, that free histamine or active H-substances were not present in such dermal fluid, such an absence of suspected offenders challenges the concept that they are entirely or chiefly responsible for the whealing reaction or, for that matter, responsible for any of the so-called "allergic" manifestations. One is still more puzzled by the fact that such whealing reactions can be alleviated, prevented, or diminished by the new antiallergic drugs known as "antihistaminic agents." What may be the mechanisms involved? We trust that some light has been shed upon this problem by the papers included in this monograph. But, in the event we find ourselves asking many more questions, most of which will remain unanswered, we may find comfort in the words of Robert Louis Stevenson, "To travel hopefully is a better thing than to arrive, and the true success is to labor."

# THE PHARMACOLOGY OF HISTAMINE: WITH A BRIEF SURVEY OF EVIDENCE FOR ITS OCCURRENCE, LIBERATION, AND PARTICIPATION IN NATURAL REACTIONS

By Sir Henry Dale

Chairman, Board of Trustees, Wellcome Physiological Research Laboratories, Kensington, London, England

It was in August, 1907, while watching a demonstration at an International Physiological Congress at Heidelberg, that I first became conscious of the existence, in certain extracts of ergot, of a potent substance different from any of those which we had hitherto encountered in that drug. Kehrer (1908), an obstetrician of Heidelberg, had been working in the department of Professor Magnus there and had found that Magnus's method of studying the reactions of surviving loops of intestine, suspended in a saline solution, was applicable to the horn of a cat's uterus. He had tested a number of substances for their action in this, including, as was most natural, a series of preparations of ergot. Of these he had found one to be pre-eminent in its prompt and powerful stimulant effect, of which he gave a striking demonstration. This was the so-called *Ergotinum dialysatum* of Wernich. Knowing something already, by that time, of the occurrence in ergot extracts of bases recognized elsewhere as products of bacterial decomposition, and finding, on inquiry, that Wernich's method of preparing his extract by dialysis was such as to provide optimal conditions for an active putrefaction, I was not inclined to accept Kehrer's conclusion that his test demonstrated the real active principle of ergot, or that it selected the extract of that drug which would be the most valuable in the clinic, with ordinary methods of administration. Indeed, it was not until 28 years later that the specific alkaloid of ergot of most value in practical obstetrics was at last discovered.

Meanwhile, in 1907, it was clear that Kehrer's test had revealed the presence, in a particular ergot extract, of something with an intense and immediate stimulant action on the tone of isolated uterine plain muscle, and different from any of the substances which had then been found in ergot. It seemed likely, on previous experience, to be an amine produced by putrefaction from some amino acid. This news I carried back from Heidelberg to London and to my late friend, George Barger. We had a batch of the *Ergotinum dialysatum* prepared for us. Our colleagues at the factory confirmed, with some emphasis, the suspicion that the process would be attended with extensive putrefaction. From it, with the guidance of my physiological tests, Barger isolated histamine (Barger and Dale, 1910). Just as we had obtained it, and before we had quite completed its identification, Ackermann (1910), of Würzburg, published an account of his preparation of histamine from histidine by deliberate putrefaction. When, eventually, we obtained a specimen from him, it enabled us to complete the identification of the base which we had isolated from the ergot extract. Then we learned that Kutscher, of Marburg, had been making the same isolation independently, at the same time; though a few comparative physi-

ological tests, which Kutscher (1910) made of his ergot base, led him to doubt its complete identity with histamine. The source of this apparent discrepancy was not difficult to discover, however, and there was soon no doubt about the identification.

In any case, histamine was not a new substance. Windaus and Vogt (1907) had already prepared it by synthesis in 1907, though they had no suspicion of its physiological activity or of its potential interest. Even the precise identity of the base from ergot was a point of no more than passing concern. The matters of real interest, and those which, by a natural development, have led to this monograph, were: the nature of the action of histamine itself as it came into view, when my late friend, Laidlaw, and I (Dale and Laidlaw, 1910, 1911) began to work on it in detail; later, its demonstrable presence in the body, apparently as a natural constituent of most living cells, but in widely different proportions in those of different tissues; and, lastly, the conditions of its release from these, in a form enabling it to produce its intense and characteristic actions on such cells as are sensitive to its effects, particularly on the cells of plain muscle and endothelia. All these belong to the pharmacological, physiological, and pathological background of this monograph on the "antihistamines." What follows is, at the sacrifice of much detail and without any attempt at completeness in crediting particular items of knowledge to their discoverers, a general survey of this background.

### *Actions and Occurrence of Histamine*

If we had dealt with its actions only as these were displayed on isolated organs, we should have described histamine simply as a powerful stimulant of involuntary muscle. Even isolated strips of any arteries which were large enough to furnish such preparations, from any of the species from which we tried them, contracted vigorously when histamine was applied in high dilutions. Yet, if histamine was injected, in doses between one half and one thousandth of a milligram, into the vein of an anesthetized cat or dog, it caused an immediate steep fall of the systemic arterial pressure, which could easily be shown by plethysmographic methods or by recording venous outflow to be due to simple vasodilatation in the limbs and in most abdominal viscera alike. The kidney alone seemed to provide an exception, since it shrank with the histamine depression more than could be accounted for by a mere passive accommodation of its volume to the general fall of arterial pressure. If, however, we took from a cat an organ which characteristically showed a vasodilatation in response to histamine thus injected into the natural circulation, whether a limb or a loop of intestine, and perfused it artificially with a saline solution or with blood, we were, for a long time, unable to demonstrate any effect of histamine but simple vasoconstriction, when it was injected into the perfusion stream in any dose which was effective at all. We had to leave that paradox unresolved for the time being, though the explanation of it, when it came some years later, provided a clue to the nature of some of the most important actions of histamine.

The actions of histamine injected into the general circulation, and especially the contrast between the syndromes which it thus produced in different species, showed, further, a most interesting and suggestive resemblance to the similarly contrasted syndromes of the anaphylactic shock, as these had then recently been described in the different species in which this reaction had been specially studied. In the anaphylactic shock as seen in the dog, a phenomenon which had first been encountered and described by Portier and Richet (1902), the central feature was a venous turgescence of the liver, with congestion of the abdominal alimentary canal and a profound collapse of the systemic arterial pressure. In a guinea pig, on the other hand, into which the second shock-releasing dose had been injected intravenously without anesthesia, though effects on the liver were not excluded, the picture of the shock was dominated by a valve-like, asphyxiating constriction of the bronchioles, leading to rapid death with acute pulmonary distention (Auer and Lewis, 1910).

Biedl and Kraus (1910) had drawn attention to the very remarkable correspondence between the effects of the well-known Witte's peptone—the product of an artificial peptic digestion of fibrin—when administered intravenously under similar conditions to the normal dog and guinea pig, and those produced in anaphylactic animals of the same two species by the injection of the sensitizing antigen. With either sensitizing antigen or peptone, the dog showed the acute hepato-enteric congestion and the profound collapse of the systemic arterial pressure, while either reagent in the guinea pig produced acute spasm of the bronchioles and death from the acute, asphyxiating distention of the lungs.

The immediate tendency had been, not unnaturally, to attribute the anaphylactic symptoms to the initiation, by the union of antigen and antibody in the blood, of a rapid proteolysis, presumably of the foreign antigen, liberating an unspecific, poisonous cleavage product acting like the familiar Witte's peptone. In the ensuing years, a relatively enormous literature accumulated, presenting a mass of detailed evidence which could be plausibly interpreted in terms of this conception of the proteolytic liberation of an "anaphylatoxin," a hypothetical "split product," formed, according to the particular variant of the theory adopted, by cleavage either of the antigen or of the proteins of the victim's own blood plasma and somehow initiated by the union of antigen and antibody. In the general examination which Laidlaw and I made of the action of histamine on these same animal species, it was impossible to miss the resemblance between the contrasted effects which this substance also produced in the dog and in the guinea pig and those produced either by Witte's peptone or by the anaphylactic reaction in the same species. Histamine again produced a profound collapse of the systemic arterial pressure in the anesthetized dog and a valve-like constriction of the bronchioles and consequent asphyxiating distention of the lungs in the unanesthetized guinea pig as the predominating features of its actions in the respective species under these conditions. In the rabbit, the correspondence seemed, for a time, to break down. Laidlaw and I had found that histamine killed the rabbit by causing an obstructive constrict-



tion of the branches of the pulmonary artery, with consequent distention and failure of the right side of the heart; whereas death of the rabbit in the anaphylactic shock had been attributed to a directly poisonous action on its heart, which we had found to be only mildly stimulated by histamine when perfused as an isolated preparation. Some years later, even this apparent exception disappeared, when Dr. Coca (1914) reinvestigated the cause of anaphylactic death in the rabbit and found it to be due, in fact, to intense pulmonary arterial constriction, causing the right side of the heart to fail from overdistention, just as Laidlaw and I had found with histamine.

The different items of evidence, as thus stated, could clearly be reconciled by identifying the anaphylatoxin with histamine, and there were not wanting those who were ready to put forward so obvious a suggestion. I am glad, on the whole, that Laidlaw and I refrained from such easy speculation, and that we were concerned rather to note the absence from the histamine syndrome of some of the characteristic features of the anaphylactic and peptone shocks. Histamine caused but a relatively mild swelling of the dog's liver, with no conspicuous congestion of the bowels and no loss of the coagulability of the blood, the latter occurring in both dog and guinea pig as a feature of the anaphylactic reaction. Even if there was indeed an anaphylatoxin—and it should be noted that there never was any really direct evidence for it—histamine could not, therefore, be identified with it. It could, at most, be only one constituent of a complex reagent, the other effects of which must be due to other constituents. We ourselves could get no further than the conclusion that the production by histamine of so large a part of the effects constituting the anaphylactic and peptone shocks must have *some* meaning, but that there were no sufficient data, at that time, even for speculation as to what that meaning might be.

Another correspondence to which we drew attention was that between the actions of histamine and those of "depressor principles," which various authors, from Vincent and Sheen (1903) onwards, had described as existing in extracts from many organs of the body and had distinguished from choline by the resistance of their effects to atropine. Popielski (1909) had postulated the existence of a widely distributed "vasodilatin" of this kind, found in organ extracts and, as he believed, also in Witte's peptone. Barger and I (1911) actually isolated histamine from an extract of fresh intestinal mucosa, in sufficient quantity to account for its atropine-resistant depressor action. The choice was, perhaps, an unfortunate one, since it was impossible to exclude completely the suspicion that histamine from such a source might be a product of bacterial action.

Let me try now to trace separately, in briefest outline, the further developments which have resulted from following the different clues presented by these preliminary studies.

*Effects of Histamine on Vascular Tone.* Take first the paradox of histamine acting as a systemic vasodilator in the cat and dog when injected into the normal circulation and as a simple vasoconstrictor on isolated arteries or artificially perfused organs from the same species. In later investigations (Dale and Richards, 1918, 1927; Burn and Dale, 1926), this

discrepancy was traced to the maintenance, under the conditions of normal circulation, of a tone of the minute blood vessels, the ultimate arterioles, and capillaries, which histamine relaxes; whereas, on somewhat larger arterial branches, its action is one of pure vasoconstriction. This tone of the minute vessels is not upheld by nervous action alone, since it is present, and is apt even to be maintained with conspicuous efficiency, in an organ deprived of its nerves by degeneration. To what constituent of the normally circulating blood it is then due is a point which is still unsettled, and one which might well be worthy of further investigation.

In our more recent experiments, with no clearly identifiable change of technique to account for it, we have found that, early even in a perfusion, with plain blood either defibrinated or kept from clotting with heparin, a tone is often built up spontaneously in the vessels of the perfused organ which, from other experiments, we can identify as a tone of the smallest vessels and which histamine then relaxes as in the normal circulation. Later in such an experiment this spontaneous tone disappears, and the vasodilator action of histamine is then replaced by the constrictor effect, which was, at one time, the only one we were able to observe with artificial perfusion. More recently, again, in unpublished experiments made during the war, I have consistently observed the appearance of this tone of the minute vessels, which histamine relaxes, in cats' legs perfused with saline solutions of hemoglobin.

In the perfused organ, this early, spontaneous tone of the minute vessels later disappears, so that histamine then causes pure vasoconstriction. A tone can then be regenerated and the dilator effect of histamine recovered by adding adrenaline (Dale and Richards, 1918) or post-pituitary vasopressin (Burn and Dale, 1926) in high dilution to the perfusing blood. Throughout the cycle of changes thus produced, it is to be noted that ordinary arterial vasodilators, like the nitrites or acetylcholine, have consistently produced only vasodilator effects, even when that of histamine has been replaced by simple vasoconstriction.

Under normal conditions, in the arterial arborization, the level at which the reaction to histamine changes from constriction to dilatation varies in the different species. In the dog, it seems to occur relatively early in the branching, so that a preparation of the mesenteric arteries can be obtained from that animal in which the tone of visible arterioles still relaxes in response to histamine. Also, the tone which histamine relaxes in perfused organs from the dog is correspondingly more resistant to the conditions of artificial perfusion. A few experiments (Burn and Dale, 1926) suggest that the level of the change in the monkey is probably also such as to include in the dilator effect of histamine arterioles larger than the most minute. In the cat, the histamine dilatation appears to be limited to the ultimate arterioles and capillaries, and in the rabbit, to be so completely confined to the capillary bed that it was only under special conditions that Feldberg (1927) found it possible to demonstrate its effect in a rabbit anesthetized for recording on the arterial pressure. Under ordinary conditions of anesthesia, as with ether or urethane, the rabbit's systemic arterial pres-

sure responds by a simple rise to any dose of histamine which is effective at all, the effect being that of a simple arterial constriction. Only under light anesthesia with chloralose is this replaced by a moderate and relatively slow depressor effect. Presumably this is due to a relaxation of a capillary tone, constituting the major component of resistance under these conditions. Its nature can be studied directly, without anesthesia, in the completely denervated ear of an albino rabbit held against the light. When a small dose of histamine is injected into a vein of the other ear of such an animal, the constriction of the arteries down to the smallest visible ones and the simultaneous flush of the tissue between them, due to dilatation of the capillaries, can readily be seen. In general terms, then, and with the differences indicated in the different species, we arrived at a conception of the histamine vasodilatation, which had presented such anomalies, as differing from other vasodilator effects only in being limited to the finer twigs of the arterial tree, if it affected the arteries at all, and in extending, in all species, to the capillary network and producing on these at least a large part of its vasodilator effect.

Experiments by Sollmann and Pilcher (1917) showed that histamine, applied to the slightly abraded human skin in dilutions down to 1 part in 100,000, causes local reddening and an urticarial wheal, suggesting that, if the capillary dilator effect had more than a minimal and transient intensity, it caused an abnormal permeability of the capillary walls. This suggestion contributed to the interpretation of the irreversible, shock-like failure of the circulation produced in the anesthetized cat by larger doses of histamine, which I was studying during the first world war with Laidlaw (Dale and Laidlaw, 1919), in continuation of the experiments with A. N. Richards. We found that, in this condition, in spite of strong contraction of the visible arteries, the circulation was failing from a deficient volume of blood in currency—an oligæmia. As much as one half the plasma had leaked through the walls of the permeable capillaries, and a large proportion of the remaining, thickened blood had become stagnant at the periphery in the relaxed capillary bed, so that the still vigorous heart had only a small volume to eject at each beat. Here, again, the total histamine effect could be interpreted as presenting, in a more intense and persistent form, the complex of vasoconstriction of all but the minutest arteries, with a general relaxation of the capillary network, involving a large increase in its effective capacity and an abnormal permeability of its endothelial walls. As might be expected, in the dog, the collapse of arterial pressure caused by histamine involved a larger component of relaxation of arterioles and a smaller component of capillary dilatation, loss of plasma, and oligæmia.

Simultaneously with these observations, Krogh (1929), by a series of elegant, direct observations, was arriving at conclusions which greatly reinforced and supplemented our own, as to the existence of a normal capillary tone keeping a large proportion of the network closed in a resting tissue, its physiological variability, and the increase of the permeability of the endothelial walls of the capillary vessels with relaxation of this tone. Sir Thomas Lewis was beginning, at the same time, his series of masterly studies

of the reactions of the small blood vessels of the human skin (later embodied in his classical monograph on that subject—1927). At an early stage of these studies, he was forced to the conclusion that the immediate, localized red reaction, followed by an oedematous wheal, which was caused by a variety of stimuli mildly injurious to the skin, was due to a chemical agent which somehow appeared as the common result of such stimuli. He inquired whether I could suggest a substance which could produce such an effect. With Sollmann and Pilcher's observations, as well as our own, freshly in mind, I supplied him with some histamine, and he found, indeed, that histamine, appropriately applied, reproduced in detail the effects in which he was interested. With exemplary caution, however, he never allowed himself to go nearer to an identification of the natural agent of the mild injury reaction than to call it "H-substance."

*Histamine and Anaphylaxis.* The early conception of the anaphylactic shock, as secondary to the digestive production of an anaphylatoxin in the blood, had been greatly weakened by physiological analysis of the phenomenon. Manwaring had shown that, in the dog, the syndrome was in the main, if not exclusively, the result of a primary action of the antigen on the liver. Such effects on the circulation as were not directly due to the sudden rise in portal pressure and acceleration of a substantial part of the blood in the viscera were clearly due to some vasodilator agent escaping from the congested liver into the general blood stream. Even the congestion of the liver and bowels were due, as we now know, to a substance causing constriction of the hepatic venous outlet, as histamine does. Then it was shown by Schultz (1910, 1912) that isolated plain muscle of the small intestine from an anaphylactic guinea pig responds by contraction to the direct application of the antigen. In experiments which I had begun independently, using the isolated uterus of such guinea pigs, I proceeded to examine this phenomenon in greater detail (1913), showing its close specificity and the possibility of reproducing all the characteristic phenomena of anaphylaxis in the guinea pig, active and passive, on preparations of isolated plain muscle from that species. There was a possibility, which must have been in many minds, that the union of antigen and antibody somehow led to that sudden appearance of histamine, whether in the dog's liver or the guinea pig's plain muscle. However, we had no evidence yet as to how that might happen and, as I pointed out at that time, the aggregating reaction of antigen on antibody might, on the other hand, merely provide us with a model, visible in a test tube, of the kind of action produced by histamine in the living cell.

I think that it was Lewis again who first gave public expression to the idea that an anaphylactic reaction, as seen in the skin, was, like other responses to mild injury, due to the appearance of H-substance. At that time I was busy, with the late Harold Dudley and with Best and Thorpe (1927), in a more critical confirmation and extension of my own early observations with Barger and the later ones of Abel and Kubota (1919), isolating histamine itself as a natural constituent of any organ which yielded an extract having this type of action—liver, lung, muscle, spleen—and in quantities

adequate to account for such activities. Thus, we were able to tell Lewis that we believed his H-substance to be histamine itself, and that we believed its appearance, in the anaphylactic or other injury reaction, to be due to the liberation of preformed, but normally bound and inactive, histamine from the injured cells and not, as Lewis had supposed, to its new formation. Lewis and Harmer (1927) and Harris (1927) then found direct experimental evidence for this view.

Thus, the relation of histamine to the anaphylactic shock started to become clear. It was made more definite by the direct evidence, produced by Gabauer-Feulnegg *et al.* (1932), of its release in the dog's liver, and by Bartosch *et al.* (1933), of its release in the guinea pig's lung, by contact of the sensitized cells, in either case, with the specific antigen. Keeping in mind the fact that there are other physiologically active components normally held, bound and inactive, in living cells, which are likely to be similarly released by anaphylactic or other cellular injury—the adenosine series, heparin, and probably others—we begin to get a reasonable picture of the anaphylactic reaction and other consequences of extensive tissue injuries and of the part played by histamine in the resulting syndromes.

That, however, does not complete the story. In 1936, Schild suspended an isolated horn of the uterus of a sensitized guinea pig and applied histamine to it in a concentration causing maximal tonic contraction. He waited until, with histamine still present, the smooth muscle eventually relaxed from this maximum to a condition of intermediate tone with a strong rhythm. A further addition of histamine to the bath then had no effect, or even caused a relaxation. An addition of the specific antigen, on the other hand, caused the sensitized uterus to contract again to the maximum, while the effect of adding more histamine was still absent or even reversed. There are, perhaps, similar discrepancies revealed by the action of some of the antihistaminic agents. Dr. de Beer, of the Research Laboratories of Burroughs Wellcome & Company, New York, sent to me, shortly before I left England, some records obtained by Mr. Castillo with a preparation consisting of a chain of isolated rings from the trachea of a sensitized guinea pig. This gives, as expected, a contraction in response to the specific antigen, the record of which is closely similar to that of one produced by histamine; but, whereas the histamine contraction is readily relaxed by benadryl, that due to the antigen is much more resistant, relaxing but slightly in response to the same agent.

An anomaly of a somewhat analogous kind was reported by Kellaway. It was known already that the rat shows a low degree of anaphylactic sensitization and that, on the other hand, the same species is highly resistant to histamine. The plain muscle of the female rat's uterus responds to ordinary doses of histamine by a weak inhibition of its tone. Kellaway found, however, that an isolated horn of the uterus from an anaphylactic rat, while responding thus to histamine by inhibition, responded to the sensitizing antigen by an augmentation of tone which, while relatively weak, was nevertheless regular and definite. It would be of interest to know whether anything can be extracted from the plain muscle of the rat's uterus, the release

of which by the anaphylactic disturbance could be responsible for a contractile response of the type observed, and, if so, whether this can be identified with any known tissue constituent. From what we know of the type of the weak response of the rat's plain muscle to histamine applied from without, we have at least no warrant for expecting, in this case, that the release of histamine in the contractile cells by an immune reaction would act as an effective stimulus.

Now, I should deprecate equally any tendency, on the one hand, to evade or slur the significance of discrepancies such as these, or, on the other hand, to regard them as immediately neutralizing all the other evidence and proving that histamine, after all, has no concern with the anaphylactic reaction in the species in which it reproduces so much of that phenomenon. Schild (1936) himself has shown that, when an isolated uterus from a sensitized guinea pig is treated with the antigen, histamine in recognizable significant amount is liberated. We have already noted the likelihood that other active constituents, normally fixed in the cell, are liberated with it. One or more of these latter may be concerned; but I am not sure that we shall need to invoke them in all these cases. Experience in another field of physiological inquiry has long convinced me that, when we are dealing with antagonisms, wide differences may be encountered between effects which are otherwise closely similar, according to whether a pharmacodynamic agent reaches the responsive cell by diffusion from without or by liberation in intimate relation to, perhaps actually within, its limiting membrane.

It has been known for many decades that, when the chorda tympani is stimulated, atropine readily stops its secretomotor, but not its concomitant vasodilator, effect on the submaxillary gland. I do not think, however, that anyone today will doubt that both effects are transmitted by the peripheral liberation of acetylcholine, presumably in relations of different intimacy to the responsive cells. Similarly, I do not think it will now be argued that vagus impulses cause contraction of the stomach wall by liberating acetylcholine and of the intestinal wall by a different mechanism, because the former effect is fairly sensitive to blockage by atropine and the latter hardly at all. Although, then, we may keep our minds still open to the possibility that all the resemblances between the effects of histamine and the symptoms of the anaphylactic reaction in different species may be misleading, I do not think that the evidence necessitates, or even justifies, any such conclusion as yet. We do not know whether the effect of histamine, when released by an aggregating immune reaction in the protoplasm of a sensitive cell, will be as readily blocked by an antagonist or by histamine itself already present in external excess, as that of additional histamine reaching the same cell by diffusion from the outside. So far as argument from analogy is justified, we should not expect the antagonism to be as effective in the former as in the latter case. And the possibility of such contrast may have an interest for the main subject of this monograph. It would not be surprising, for example, to hear evidence that an antihistaminic remedy is less effective against a bronchial spasm than against a vascular reaction in skin or mucous membrane.

*Summary*

Though histamine is a stimulant of nearly all plain muscle, the intensity of its action is not uniformly distributed. The plain muscle coats of the bronchioles and of the uterus, especially in the guinea pig, seem to be especially responsive to its action. That of the guinea pig's ileum is also exquisitely sensitive. This, with its relaxing effect on capillary walls, seems to account well enough for the manifestations of its actions in the asthmatic and urticarial reactions of the allergies. Additional effects, not obviously connected with these two main actions, are the stimulation of gastric secretion, the production of severe headache, and a rapid fall of the body temperature. Apart from its release by allergies and other injurious reactions, it is released, in quantities sufficient to produce general vasodilator reactions and gastric secretion by certain substances acting as "histagogues," by curarine (Alam, Anrep, Barsoum, Talaat, and Wieninger, 1939) and certain diamidino and diguanidino derivatives as well as by licheniformin, an antibiotic (MacIntosh and Paton, 1947).

There remains a question of great physiological interest: whether and, if at all, to what extent the vasodilator action of histamine participates in the functional adjustment of the circulation to metabolic need. Since Gaskell (1877) first put forward the suggestion seventy years ago, there has accumulated a large volume of evidence for a predominant role of substances liberated from the cells of a tissue during active function, in the dilatation of its blood vessels, often long outlasting the phase of activity. The substances concerned have often been spoken of collectively as "metabolites"—a term which is unsatisfactory, not only as being vague, but as implying, at least in advance of the evidence, that the substance or substances in question are actually products of, and not merely that their appearance is a concomitant of, the accelerated metabolism.

In the Herter Lectures (Dale, 1920) delivered on the occasion of my first visit to the United States, I discussed in some detail the possibility that histamine might at least participate in the production of this functional hyperaemia. I pointed out that the predominant incidence of its vasodilator action on the smallest blood vessels, and particularly on the capillaries, should render it an ideal agent for the fine adjustment of the circulation to varying, local, metabolic demands, enabling it to shift the prevailing course of the blood flow from one mesh to another of the capillary network as one cell or tissue unit after another became involved in activity. I was obliged, however, to admit that such speculations would have no basis but imagination, unless we could obtain direct evidence to justify the assumption that histamine is, in fact, liberated from cells which enter into functional activity.

The position has changed in more than one respect in the 29 years which have intervened. We know now that most, if not all, of the body cells which exhibit a vigorous metabolism do contain histamine as a normal constituent of their protoplasm. We know also, especially from the work of Lewis and his school, that stimuli applied to such cells, if they are vigorous enough to entail even mild injury, do, in fact, cause this histamine to be liberated from them in sufficient amounts to cause dilatation of the adjacent capil-

laries, to a degree which can be regarded as mildly pathological. For direct evidence of the liberation of histamine by normal cellular activity, in a manner adequate to account for functional vasodilatation, we seem as yet to be limited to the highly suggestive experiments of Anrep and his co-workers. Anrep and Barsoum (1935) and Anrep, Barsoum, Talaat, and Wieninger (1939) found that contraction of voluntary muscle in the dog, whether evoked by artificial stimulation of the nerve or by centrally convulsant drugs, in the normally innervated muscle, or by direct stimulation of the muscle denervated by degeneration, is accompanied by an output of histamine, appearing in the venous blood from the muscle as long as the hyperemia continues, which is evoked by and continues after the contractile activity.

The experimental findings presented by Anrep and his colleagues appear to be so clear cut and regular, and their significance, if established beyond question, seems likely to have wider applications of such importance that it is to be hoped that confirmation and extension of them may soon be forthcoming from other laboratories. If the functional liberation of histamine is to be accepted, the question still remains whether it acts by itself or by a synergism with  $\text{CO}_2$ , a possibility suggested by observations recently shown to me by H. Rein, in Göttingen. Further, and as a point which seems to me to have special relevance to the subject of this monograph, if histamine does in fact, and to any degree, play an essential part in such a fundamentally important physiological mechanism of adjustment, we must surely keep that function in mind in considering the actions and the desirable applications of the antihistaminic remedies. We ought, perhaps, to seek some assurance that the use of these substances to give relief from distressing and deleterious symptoms, due to pathologically liberated histamine, does not entail the harmful suppression of a part played by histamine in a normal and essentially physiological adjustment. One should hesitate, in default of direct and compelling evidence, to proceed on the assumption that the natural occurrence of histamine, in the active cells of so many organs and tissues, has none but a potentially injurious significance.

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# THE PHARMACODYNAMICS OF HISTAMINE

By Bradford N. Craver

*Research Division, Ciba Pharmaceutical Products, Inc., Summit, New Jersey*

To make understandable the case history, as it were, of allergy, one must be familiar with the characteristics of the "skeleton in the closet." Histamine plays the role of such a "skeleton" in relation to allergy. Since the papers dealing with the peculiar problems of allergy have clarified this relationship, we shall confine ourselves purely to the characteristics of that "skeleton," save for minimal references to the earliest papers suggesting a connection between allergy and histamine.

Histamine was first synthesized in 1907 by Windaus and Vogt.<sup>1</sup> In 1909, Biedl and Kraus<sup>2</sup> advanced the hypothesis, based upon their studies of peptone-shock and anaphylaxis, that the symptoms resulting therefrom might be attributable to the release of a toxic substance within the organism. In 1910, Kutscher<sup>3</sup> and Barger and Dale<sup>4</sup> announced their independent isolation of histamine from ergot. In the same year, Dale and Laidlaw,<sup>5</sup> after a careful study of the physiological effects of histamine, suggested that this substance might be responsible for the symptoms of peptone-shock. The comprehensive investigations of Dale and Laidlaw concerning the physiological effects of histamine clearly delineated the major actions of the drug.

The hundreds of papers concerned with histamine that have been published since that time have added details to the picture sketched by Dale and Laidlaw. We can consider the present state of that picture, but it would be impossible to trace the history of every detail. Such histories are readily available in the two able monographs by Feldberg and Schilf<sup>6</sup> and by Gaddum and Dale<sup>7</sup> and in the four excellent reviews by Best and McHenry,<sup>8</sup> Rose,<sup>9</sup> Dragstedt,<sup>10</sup> and Selle.<sup>11</sup> Of the many investigations in recent years that have been concerned primarily with the metabolism of histamine, only a partial review will be possible.

Histamine is readily absorbed after injection and rapidly exerts its effects. Histamine administered enterally is slowly and poorly absorbed, although slight absorption does occur from the small intestine.<sup>12</sup> Histamine must be reasonably well absorbed from the oral cavity, since, when administered lingually, it has been lethal to guinea pigs.<sup>13</sup>

Best and his associates<sup>14</sup> first definitely established the presence of histamine in most, if not all, tissues of the body. Earlier workers had isolated histamine from various tissues,<sup>15</sup> but the demonstration by Ackermann<sup>16</sup> in 1910 that bacteria could produce histamine from histidine had raised the question of whether the histamine subsequently isolated was a natural constituent of the tissues or was produced by decomposition. The analytical method devised by Best and his associates eliminated this doubt. In general, its concentration is particularly high in lung, intestine, and skin and usually particularly low in the blood and kidneys; but there are considerable variations among species.

In 1935, Barsoum and Gaddum<sup>17</sup> devised a more sensitive analytical procedure. It still employed, however, essentially the same principles incor-

porated in the method of Best and his associates. Code<sup>18</sup> added to their procedure minor changes that served to make it more practicable. Using his modified procedure, Code and others have confirmed the earlier results.

Histamine injected intravenously rapidly leaves the blood stream.<sup>9</sup> The reason for this is not known, since, to date, the only enzyme specifically implicated in the detoxification of histamine is histaminase, first isolated by Best and McHenry<sup>19</sup> and later studied by them in detail. The existence of such an enzyme had been foreshadowed by the report of Eustis<sup>20</sup> in 1915 that histamine was inactivated after incubation with buzzard's liver. This enzyme has since been shown, by Zeller and his associates,<sup>21</sup> to be a relatively unspecific diamine oxydase. This enzyme acts *in vitro* very slowly, whereas the detoxification of histamine *in vivo* is very rapid. This suggests one of two possibilities: (1) either other enzymes are concerned with the detoxification of histamine *in vivo*; or (2) there are present, *in vivo*, substances that markedly accelerate the action of histaminase.

Rocha e Silva<sup>22</sup> has suggested that histamine may be temporarily inactivated by combination with the free carboxyl groups of amino acids within the cells. There is also evidence that certain non-specific detoxifying agents, such as ascorbic acid, glycine, glucuronic acid, and cystine, may play a role,<sup>23</sup> a possibility strengthened by the report that the administration of histamine would increase the urinary excretion in guinea pigs of combined ascorbic acid.<sup>24</sup> Some histamine is excreted by way of the urine, from which it was isolated by Ackermann and Fuchs.<sup>25</sup> Anrep and associates<sup>26</sup> showed that it is excreted in both a free and combined form, with the combined form predominant in Carnivora and the free form in Herbivora. The administration of histidine has been reported, by Holtz and Credner,<sup>27</sup> to increase the urinary excretion of histamine. This is reasonable, since histidine by decarboxylation within the body gives rise to histamine. Alexander<sup>28</sup> reported that urinary excretion in mice tended to parallel the total concentration of histamine in the body. It is certainly clear that urinary excretion is not a major method by which the body eliminates excessive histamine. Histamine has also been isolated from feces by Myhrman,<sup>29</sup> from menstrual fluid by Gibertini,<sup>30</sup> and from human milk by De Toni.<sup>31</sup>

The problem of tachyphylaxis and tolerance to histamine has been too recently reviewed by Wells, Gray, and Dragstedt<sup>32</sup> to justify its repetition. They found no evidence for either phenomenon, and, in several hundred experiments in our laboratories with dogs with Thiry-Vella loops that received many injections of histamine at rather frequent intervals, we have seen neither phenomenon. Cantoni<sup>33</sup> recently reported that the tachyphylaxis to large doses of histamine originally reported by Barsoum and Gadum<sup>17</sup> can be eliminated in *in vitro* experiments by increasing the concentration of potassium in the perfusion fluid. One cannot deny that others have found evidences of both tolerance and tachyphylaxis. It is obvious that the conditions of the experiments could not have been the same. It re-emphasizes the need for standard conditions in investigating the phenomena of tolerance and tachyphylaxis.

Three main physiological actions account for most of the effects of paren-

terally administered histamine: (1) its stimulating action upon smooth muscle; (2) its dilating action upon capillaries, which is extended in some species to arterioles (other portions of the vascular tree are, in general, constricted); and (3) its stimulating action upon glands, including the gastric glands and the adrenal medulla.

The stimulating action upon smooth muscles includes almost all those that might be mentioned—the bronchial muscles, intestinal, splenic, vesical, those of the gall bladder, small intestine and colon, uterus, vas deferens, and epididymis.

The sensitivity of various organs and of the same organ in different species differs greatly. These wide variations in sensitivity, as well as certain anomalous results, present challenging problems to a solution of which our present knowledge is unfortunately unable to contribute. Thus, the lapin ileum responds, *in vitro*, only to a dose several hundred times that needed to cause an excellent contraction of the ileum of the guinea pig. The uteri of all species studied are contracted by histamine, save that of the rat, which is relaxed. A more extensive list of these peculiarities would be uninspiring and useless, since any principle that would permit correlation has thus far eluded us.

The action of histamine upon the blood vessels accounts for many of the symptoms that its injection can produce. The capillaries are dilated, a presumed direct effect unaltered by atropine. Knox,<sup>34</sup> however, recently reported that the enhanced dilatation caused by histamine of the vessels in the leg of the cat perfused with a calcium-free fluid could be abolished by atropine. The arteries and veins are contracted by histamine. The action upon the arterioles varies with the species and tends, in the highest mammals, to be dilatation and, in the lowest mammals, to be constriction. Once again, we encounter unexplained exceptions. Thus, Woodbury and Hamilton<sup>35</sup> concluded that histamine did not constrict the pulmonary vessels of the dog as it did in the cat and rabbit, and the experiments of Friedberg, Katz, and Steinitz<sup>36</sup> confirmed their results. The net effect of histamine in any species upon the blood pressure, which is the algebraic sum of many variables, will depend considerably upon the relative sensitivity of the various portions of its vascular system. Thus, in man and monkey, whose capillaries and arterioles are dilated by histamine, a hypotension results from the administration of the drug; whereas in the rabbit, whose arterioles are constricted and only the capillaries dilated, a hypertension is seen.

Histamine has a significant stimulating action upon many glands. Dale and Laidlaw<sup>5</sup> first remarked this in relation to the lachrymal, bronchial, salivary, and pancreatic. The remarkable stimulating action of histamine upon the gastric glands was first observed by Popielski<sup>37</sup> in 1920. This has since been intensively investigated. The controversy, as to whether gastric secretin is histamine, has not yet been satisfactorily settled. The difficulties that must be resolved in order to answer this problem have been discussed by Emmelin,<sup>38</sup> who favors the view that gastric secretin is not histamine, but depends for its action upon a liberation of histamine in the gastric mucosa. Ackermann,<sup>39</sup> in a careful review of the physiology and pharmacology of the

sweat glands, concluded that histamine had little effect thereon. The action of histamine upon glands presents many interesting problems which, unfortunately, we do not have the space to consider.

The observations of Gibbs and McClanahan,<sup>40</sup> that atropine would inhibit the sialogogic effect of histamine, has been confirmed many times. Cornil<sup>41</sup> reported that, in the curarized dog, the stimulation of histamine upon the salivary glands was more pronounced. This recalls the observation by Alam, Anrep, Barsoum, *et al.*<sup>42</sup> that curare caused a relatively marked increase in the blood histamine.

Injections of histamine have been reported to cause an increase in the pressure of the cerebral spinal fluid. Such a rise was reported by Weiss and his associates<sup>43</sup> and was later confirmed by Friedman<sup>44</sup> and others. A similar response was reported for the dog. Recent experiments in our own laboratory, however, in which the cerebral spinal fluid pressure has been continuously recorded, have indicated that one may see a rise, a fall, or a bi-phasic reaction in which an initial fall is followed by a secondary rise. The last reaction is the most common. These variations are not surprising when one considers that the cerebral spinal fluid pressure is as much the algebraic sum of many variables as is the blood pressure. The initial fall probably represents a decreased circulation to the brain as a result of the sudden hypotension; a secondary rise probably reflects the secondary cerebral vascular congestion; and the slow but continuous rise that may be seen in some experiments after repeated doses of histamine may reflect some increase in the permeability of the choroid plexus.

The actions of histamine upon the heart are of interest, although the results reported are not entirely in accord. Lissak and Kokas<sup>45</sup> reported that histamine had a positive inotropic effect on the isolated spring frog heart, with a negative effect upon the winter frog heart. He attributed this to the level of metabolism, since thyroidectomy of the spring frogs reversed the positive inotropic effect. These effects were confirmed by Martin.<sup>46</sup> The effects of histamine upon the human heart were studied with electrocardiograms by Massione and Picchio,<sup>47</sup> by Weiss and his associates,<sup>48</sup> and more recently by Albers and Petzold.<sup>49</sup> They concluded that the abnormalities observed reflected significant toxic effects. Peters and Horton,<sup>50</sup> however, made similar studies during a constant infusion of histamine and considered the electrocardiographic changes, the most common of which was the loss or inversion of the T wave, of minor consequence, especially since they disappeared shortly after the discontinuance of the infusion. Hueper and Ichniowsky<sup>51</sup> made extensive pathological studies of dogs that had survived prolonged and severe histamine shock. They found serious and irreversible changes in the myocardium, as well as in many other tissues. These changes were probably not direct effects of the drug but were rather the results of the shock induced by the histamine.

The lymphogogic action of histamine, initially reported in dogs by McCallell and Drinker,<sup>52</sup> has since been confirmed by several other investigators. This has been considered secondary to the general vascular changes, in particular, the increased pressure in the portal system.

The relation between the adrenals and the physiological actions of histamine has received such intensive study that it would be impossible to attempt more than a summary of the results. In 1920, Dale<sup>53</sup> reported evidence that histamine increased the output of the adrenal medulla, but it was not until 1926, when Burn and Dale<sup>54</sup> were able to show that the secondary rise in blood pressure in cats following the injection of histamine could be eliminated by removal of the adrenals, that definite evidence for the relationship between the two was available.

In 1926, Banting and Gairns<sup>55</sup> first demonstrated that, in dogs, adrenalectomy caused a remarkable increase in the animals' sensitivity to histamine. Wyman, in 1928,<sup>56</sup> confirmed this with rats and showed that their sensitivity could be decreased by an intraperitoneal injection of epinephrine. Perla and Gottesman<sup>57</sup> were not able completely to protect their rats by epinephrine. In 1937, Ingel<sup>58</sup> sought to restore the resistance of adrenalectomized rats to histamine by the administration of cortin, but he was unable to do so until the cortin had been supplemented by epinephrine. Rose<sup>9</sup> has shown that the rat's capacity to detoxify injected histamine is impaired by adrenalectomy. He has also shown that very large doses of cortin would decrease the rat's sensitivity to normal levels. It would appear from these and other results that the increased sensitivity of adrenalectomized rats to histamine is due to a loss of both secretions of the adrenals. The recent report by Staub<sup>59</sup> that therapeutic doses of epinephrine injected into patients immediately and briefly increases the blood histamine is most interesting, but serves, at the moment, to increase the problems of interpretation. Burn and Dale<sup>60</sup> had suggested such a possibility, but Dale and Richards<sup>61</sup> were unable to demonstrate it. It remained for Eichler and his associates<sup>62</sup> to prove it occurred, although they employed nonphysiological doses in cats (as high as 3.0 mg/kg).

Thus far, attempts to explain the normal functions of histamine within the organism have been largely unsuccessful. Its marked oxytocic action has tempted efforts to inculcate it not only in the onset of normal labor, but in the toxemias of pregnancy as well. Such studies were initiated by Kapeller and Adler<sup>63</sup> and have been extended by Werle and Effkemann<sup>64</sup> and others. They have indicated that the blood content of histaminase increases in normal pregnancies, and that, in pathological pregnancies, abnormalities in the metabolism of both histidine and histamine can be detected. The significance of these changes remains obscure. Kwiatkowski<sup>65</sup> has presented evidence that nervous stimulation may cause a release of histamine in certain tissues. This would provide us with a class of histaminergic nerves. Rosenthal and Minard<sup>66</sup> advanced the theory that histamine was the chemical mediator for nerves transmitting sensations of pain. The marked local anesthetic action of the antihistaminics now clinically employed offers indirect support for their hypothesis. Whether the anti-histaminic and local anesthetic actions of those drugs are separate, unrelated attributes or merely divergent expressions of the same fundamental action has not yet been determined.

It has been shown by Anrep and his associates,<sup>67</sup> Ambache,<sup>68</sup> Marcu,<sup>69</sup>

and others that all three types of muscles—skeletal, cardiac, and smooth—liberate histamine during activity. No explanation is available for this interesting but as yet unconfirmed finding. The problems of the physiological functions of histamine must await further research for solution. It is certain that histamine must fit into something more than a theory of allergy.

In conclusion, a brief discussion of the mechanism of histamine's action would appear relevant. Histamine has been classically considered a myotropic stimulant. Somewhat over two years ago, in our laboratories, a study was initiated of the relative potency of various antihistaminics in nullifying the intestinal spasm induced by injected histamine in dogs with Thiry-Vella loops. Anomalous responses to histamine began to appear

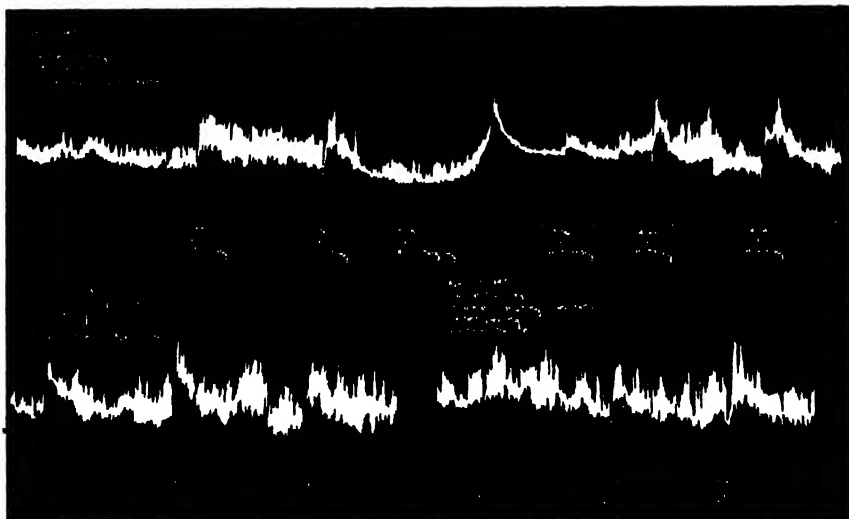


FIGURE 1. April 7, 1947. In this record the stimulating action of di-isopropylfluorophosphate upon the intestine is evident, as is also its potentiation of the action of subsequently injected histamine. The lower left-hand record, secured in an experiment on the same dog performed three days after the injection of DFP, shows some persistence of the potentiating action. In the lower right-hand record, secured in an experiment performed on the same dog, ten days after the injection of DFP, the potentiating action is lacking.

which the classical theory could not explain. Early in 1946, Ambache<sup>70</sup> advanced the hypothesis and offered persuasive evidence therefor that histamine acts by causing a liberation of acetylcholine within the body. Since this hypothesis explained the peculiar results we had obtained, we sought to repeat his experiments in part and to test the validity of his hypothesis by other methods.

We attempted to demonstrate that sections of the small intestine of the rabbit or cat would, after a variable number of days of cooling in Locke's solution, attain a state in which they had lost all responsiveness to histamine but would still respond to relatively large doses of acetylcholine. Ambache had demonstrated such a state with the intestines of the rabbit and guinea pig. His explanation thereof was that the tissues had lost their capacity to produce acetylcholine and hence would no longer react to histamine, the effect of which depended upon an endogenous release of acetylcholine.

Since the muscular mechanism remained at least partially functional after the degeneration of all nervous tissue, added acetylcholine still worked.

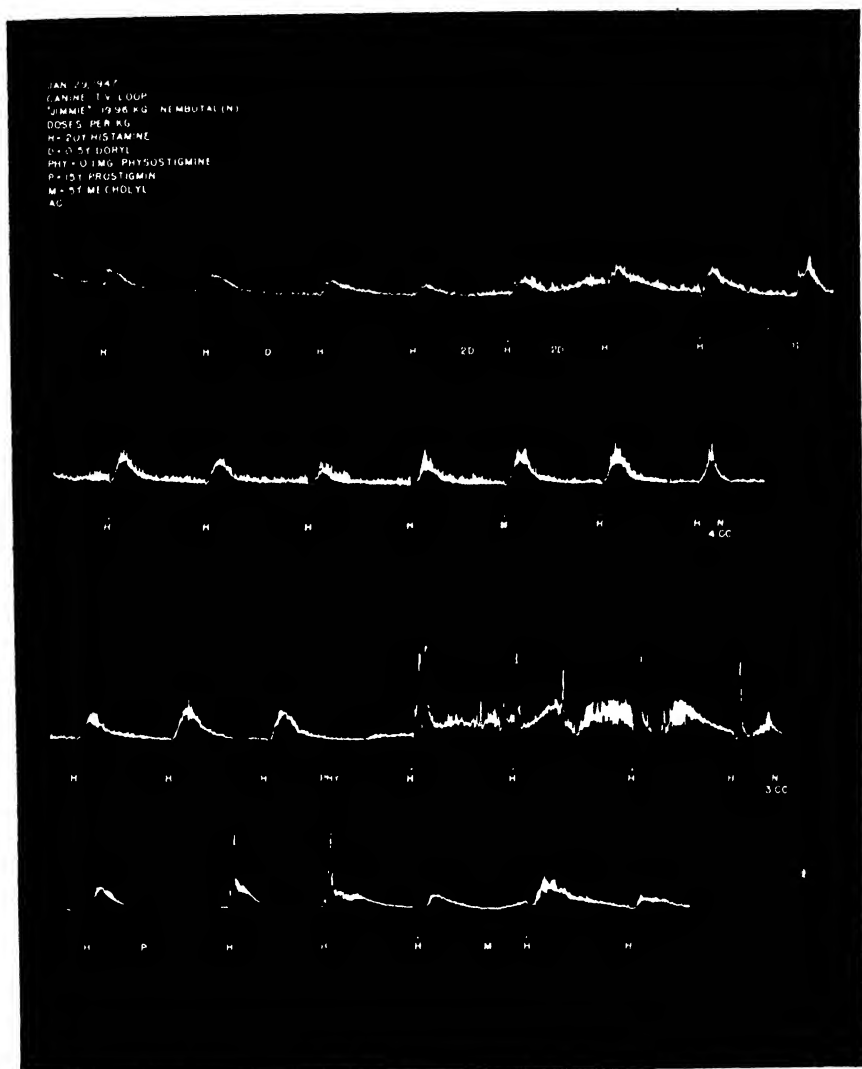


FIGURE 2. January 29, 1947. The marked potentiation of histamine's action by physostigmine and prostigmine is evident. Doryl and Mecholyl may be seen to have caused little or no potentiation.

Unfortunately, in our own experiments, we found the responses to histamine and acetylcholine disappeared *pari passu*.<sup>71</sup> Although conditions for our experiments were similar, thus far, the cause of the discrepancy has eluded us. It was possible to show that the intestinal spasm induced in Thiry-Vella loops by intravenously injected histamine could be potentiated



by di-isopropylfluorophosphate, physostigmine, and prostigmine, the actions of which depend primarily at least upon an inhibition of cholinesterase, but not by Mecholyl or Doryl, the chief mode of action of which is different. The histamine-induced intestinal spasm was accentuated and prolonged in adrenalectomized dogs. This was expected, because histamine has been shown, since the first suggestion by Dale in 1920,<sup>73</sup> to increase the adrenal's output of epinephrine, whose actions antagonize those of histamine. This effect might be due to a release of acetylcholine at the preganglionic neural endings within the adrenal medulla.

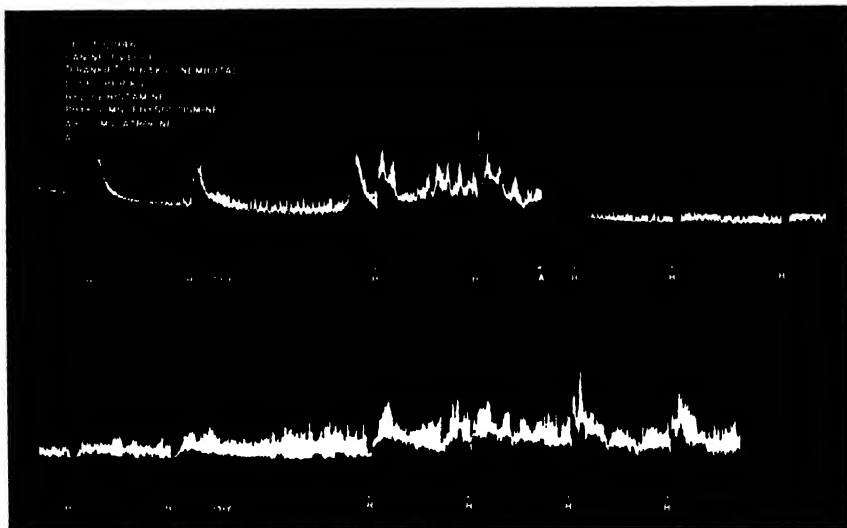


FIGURE 3, December 30, 1946. The record reveals the bi-phasic nature of the intestine's response to histamine. After atropine, the stimulating action of histamine is absent and its inhibitory action marked. The antihistaminic activity of atropine may be seen as well as the potentiation of histamine's action by physostigmine.

The action of histamine upon the intestine was shown to be biphasic with an initial, and at times scarcely discernible, inhibition which was quickly followed by marked stimulation. After the administration of atropine, the stimulation was absent and the inhibition more marked. A possible explanation might be the liberation of acetylcholine at preganglionic sympathetic endings, since atropine is considered to negate the effects of acetylcholine only at the post-ganglionic endings of the parasympathetic nerves.

These data strengthen Ambache's hypothesis, but it is by no means as yet a theory. It is certain that the pharmacological actions of acetylcholine and histamine are very similar. There are, however, disturbing discrepancies. To mention but two: (1) acetylcholine contracts the uterus of the rat, but histamine does not;<sup>73</sup> (2) acetylcholine dilates arterioles, but histamine generally constricts them.<sup>9</sup> It has become increasingly apparent that intimate relations exist amongst the three powerful physiological agents acetylcholine, epinephrine, and histamine. The future yet holds the interesting day when their biographies become completely known.

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# THE ROLE OF HISTAMINE AND OTHER METABOLITES IN ANAPHYLAXIS

By Carl A. Dragstedt

*Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois*

To discuss the role of histamine and other metabolites in anaphylaxis as a part of this monograph is to risk the Scylla of a burdensome repetition of an oft-told tale and the Charybdis of inexcusable neglect, since the principal topic, "Antihistamine Agents in Allergy," traces its genealogy to this experimental problem. I shall try neither to bore you with all the oft-quoted evidence for the role of these agents in anaphylaxis nor to evade my responsibility in clarifying the background for this monograph and the corresponding background for the current approach in the therapy of allergy.

The prevalent theory as to the pathogenesis of the varied symptoms appearing during anaphylactic shock holds that these symptoms are mediated by histamine and other tissue metabolites which are liberated in some manner as a consequence of the antigen-antibody reaction. If this is viewed as a theory, subject therefore to amendment, support, or refutation, it seems not inappropriate to survey briefly the various theories which preceded it, for the *post-mortem* analysis of deceased theories serves to emphasize the criteria by which we may assess the frailty or strength of a current postulate.

There are a number of theories that require no more than mention, for the assemblage of facts which has destroyed their tenability is well known to all. Among such are the theories that the anaphylactic symptomatology may be ascribed (1) to an excitation of an immunity center in the central nervous system, (2) to intravascular coagulation and embolism, (3) to esoteric disturbances in the colloidal balance of the circulating plasma, (4) to altered viscosities in the blood and muscle plasma, (5) and to the toxic half of the antigen, envisaged as a sort of Siamese-twin union of toxic and nontoxic units.

Worthy of more extended mention, of theories respecting the mechanism of the anaphylactic reaction, was the crystallizing of ideas into what were called the anaphylatoxin or humoral hypothesis, on the one hand, and the cellular theory on the other. Taking origin in, or at least a hint from, Pfeiffer's original endotoxin theory as to the pathogenesis of symptoms in infectious diseases, and receiving varying kinds of support from the observations of Wolff-Eisner, Friedberger, Vaughan, and Wheeler and others, the general tenor of this anaphylatoxin hypothesis was to the effect that anaphylactic shock is a true intoxication due to a poison produced from the products of the antigen-antibody reaction by the action of complement, the reaction occurring intravascularly.

Contrariwise, the observations of Manwaring, Pearce and Eisenbrey, Coca, Weil, Schultz, Dale, and others appeared, presenting such unequivocal support to the rival cellular theory that, in 1923, Zinsser summarized the situation as follows: "There is, thus, an incontrovertible mass of evidence available which proves without question that the site of the reaction, which

carries in its train the symptoms which we speak of as anaphylaxis, is predominantly on the cells of the body."

This verdict, in view of the irreconcilability of the opposing theories, was, thus, a death sentence to the anaphylatoxin hypothesis. This was regrettable in that the anaphylatoxin hypothesis, in addition to speculating as to the site of the reaction, presented an intelligible explanation of the ensuing phenomena, while the cellular theory was at that time restricted to localizing the site of the reaction and left largely to the imagination the mechanism by which a cellular reaction could induce the characteristic phenomena of anaphylaxis.

Meanwhile, the observations of Dale and Laidlaw on the pharmacological effects of histamine, of Barger and Dale and of Best, Dale, Dudley, and Thorpe on the presence of histamine in animal tissues, and of Lewis on various human skin reactions, paved the way for Dale's extension of the cellular theory, which, although phrased in 1929, is still such a cogent statement of our current view that it demands repetition. He said, "We may picture the anaphylactic shock, therefore, as the result of cellular injury, due to the intracellular reaction of the antigen with an aggregating antibody. Whether this is general or localized in a particular organ, histamine will be released and its effects will be prominent in the resulting reaction, imposing a general resemblance to the syndrome produced by histamine itself, on the symptoms seen in each species. The cell injury, however, is not limited to the degree required to produce a release of histamine, and involves other and more direct results. Such a conception is in accordance with all the facts as yet available, and it has the advantage of rendering intelligible, not only the striking resemblance between symptoms of the anaphylactic reaction and those produced by injecting histamine, but also the various and equally significant points of difference between the two syndromes."

In 1932, however, after the first experimental evidence directly supporting Dale's theory had been reported, an editorial commentator wrote as follows: "However, the existence of a secondary allergic toxic factor can no longer be doubted if one is to credit the current biochemical studies by Dragstedt and Gebauer-Fuelnegg of Northwestern University Medical School, and the simultaneously reported anaphylatoxin studies by Bartosch, Feldberg, and Nagel of the physiologic institute at the University of Berlin." This maladroit employment of the term "anaphylatoxin," with reference to our observations on the release of histamine in anaphylaxis, seemed to imply that there was an attempt at a reincarnation of the defunct anaphylatoxin hypothesis and thereby an attempt to discredit the cellular theory, for, as Zinsser had concluded, "All workers who have inclined to an anaphylatoxin theory have necessarily assumed the site of the reaction to be in the circulation."

Except for the borrowing of this euphonious term from the opposing anaphylatoxin theory, there is no conflict whatsoever between Dale's histamine theory, with the experimental evidence supporting it, and the so-called cellular theory. Dale's statement and all of the experimental evidence acquired during the past fifteen years are only extensions of the cellular

theory and have clothed the cellular theory with meaning and verified its validity. This point cannot be stressed too forcefully, because I am convinced that almost all the opponents of the histamine theory base their opposition upon a misunderstanding of this fact. For example, a recent reviewer writes as follows: "Some of these objections, however, have been adequately met by more recent findings; others have not, and until they are all satisfactorily answered, the histamine hypothesis cannot take precedence over the cellular theory."

Viewing the anaphylactic reaction as a special form of cellular injury which permits the release of cellular metabolites, we are concerned with those metabolites which, by virtue of having physiological activity, might impose their effects upon the diverse pattern of the reaction. The list would include adenosine, acetylcholine, heparin, histamine, lysocithin, potassium, various intracellular enzymes, and probably others.

The problem of assessing the role of these agents in the anaphylactic reaction becomes one of cataloguing the anaphylactic symptomatology, identifying one or more of these metabolites in a causal relationship to one or more symptom, and determining how well we can balance the accounts. Of paramount importance is the necessity of a quantitative evaluation. One of my late professors always cautioned his students about the fly that sat on the axle of the speeding chariot wheel and bragged of the dust he was raising. Thus, the mere recognition of one of these metabolites in association with the anaphylactic reaction does not distinguish it as a participant rather than a spectator in the performance, although one would have to grant that its peculiar properties, viewed in the light of the nature of the anaphylactic reaction with which it was associated, might give good grounds for suspicion.

Probably the clearest case of all has been made with respect to the role of heparin. Since Jaques and Waters identified, isolated, and quantitated heparin in the blood of anaphylactic dogs, there has been no question as to its role in the incoagulability of the blood in this animal. Although incoagulability of the blood is a less conspicuous phenomenon in the anaphylactic reaction of other animals, there is good ground for assuming that it might account for such changes as do occur. Parenthetically, it seems odd that there have been no studies reported, so far as I am aware, as to whether heparin does, or does not, appear in human blood during severe allergic reactions. Correspondingly, it seems odd that the opponents of the histamine theory have not had recourse to considering the absence of such evidence in the human reaction either as an argument against the theory, as applied to anaphylaxis on the one hand, or as an argument against the identity of the anaphylactic reactions of animals with the allergic reactions of man on the other.

Acetylcholine, adenosine, choline, lysocithin, potassium, the slow-reacting substance of Kellaway and Feldberg, and a proteolytic enzyme have all been identified, more or less definitely, in connection with anaphylactic reactions. The indictment of them as participating agents in the reaction has not as yet progressed much beyond the recognition that they might play

some role, nor have they been fully exonerated by any evidence which would preclude their participation. At the moment, their identification serves to indicate the complex character of the cellular injury involved in the anaphylactic reaction and to suggest potential mechanisms which may explain and clarify some of the symptoms of anaphylaxis not otherwise accounted for.

With respect to histamine, its status as a participating factor in the anaphylactic reaction seems to be midway between that of all these agents, which as yet are only suspect, and that of heparin, which may be considered unequivocal. In thus abridging the status of the role of histamine, it is not intended that its importance be in any way minimized.

Consider for a moment the nature of the evidence implicating histamine. In the dog, histamine has been shown to leave the liver and to appear in the blood and lymph, both in such quantity and with such explosive rapidity as to permit no reasonable alternative to its participation in the reaction. The evidence is essentially as conclusive for the guinea pig, although the source of the histamine in this animal is more pulmonary than hepatic. Since the administration of histamine, in doses equivalent to those estimated as released in anaphylaxis, has serious to fatal effects in these animals, one cannot deny that it has a major role in the anaphylactic reactions of these animals, even though evidence to preclude the collaboration of other factors is incomplete. The role of histamine in the anaphylactic reaction of the rabbit is not so clear.

There is no doubt that histamine is released from the cellular elements of the blood into the plasma, thereby providing the opportunity for it to become physiologically active. There is no doubt that this release occurs with sufficient rapidity. As yet, however, we do not have adequate data of a quantitative character to permit an evaluation of the importance of this histamine release, and we do have some evidence that obstruction to the pulmonary circulation, independent of histamine, may have significance. Thus, the status of the role of histamine in anaphylaxis is more ambiguous than that of heparin, in that the collaboration of other agents or factors cannot be excluded so readily. Its importance, however, is far greater. The discharge of heparin does not appear to jeopardize either the life or health of the animal and it is not known to be concerned in any important phase of the reaction other than that of the coagulability of the blood. Histamine, however, is important by virtue of the serious import of its effects as well as by its ability to impose its action in such diverse fashion.

To draw up a provisional balance of accounts between the varied symptoms of anaphylaxis on the one hand and the tissue metabolite most probably concerned in its pathogenesis on the other, it is clear that heparin can account for the incoagulability of the blood and perhaps for some minor phenomena, such as the anticomplementary titer of the blood. It is also clear that histamine can account for the majority of the smooth muscle reactions, glandular secretions, increased lymph flow, and vascular phenomena. This leaves some characteristic features of the anaphylactic reaction unaccounted for, such as the necrosis of tissue as seen in the Arthus' phenomenon; *etc.* To what extent the agents other than heparin and his-

tamine, which have been identified with the reaction, contribute to the syndrome is not yet clear.

The proponents of the original anaphylatoxin hypothesis devoted a great deal of effort to finding some unitarian toxin which would have all of the diverse effects which are manifest in the anaphylactic reaction. The opponents of the modern extended version of the cellular theory seem to have been converted to this unitarian doctrine, in that they are inclined to impeach any tissue metabolite identified with anaphylactic shock on the basis of the incompleteness with which it mimics every item in the category of anaphylactic symptomatology. As it happens, they have directed their criticism at histamine and not at all at heparin. I do not understand the logic of this attitude. That histamine may have less significance in the reaction of other animals than in that of the dog and guinea pig is of no more moment than the fact that heparin has less significance in other animals than it has in the dog. Nor, to generalize, is there good reason why the other tissue metabolites associated with the phenomena of anaphylaxis should have parallel degrees of importance in more than one animal species.

With respect to the present monograph, the role of histamine and other metabolites in anaphylaxis has significance only to the extent that the problem of human allergy has kinship with the problem of anaphylaxis and to the extent that the agents shown to be of importance in the one case are also shown to be of importance in the other. It is not in my province to discuss this question, and I shall merely comment that it seems strange that one of the opponents of the histamine theory should have written a paper in 1930 defending the fundamental identity of animal anaphylaxis and human allergy, but now, though admitting the causal relationship of histamine to anaphylaxis in the dog and guinea pig, discredits its possible role in human allergy because it has not yet been proven to be of paramount importance in the rabbit, horse, and calf. I do not believe that an argument on the basis of phylogenetic relationship is any better in one case than it is in the other.

In conclusion, I beg your indulgence for a parody on Kipling's immortal eulogy to Gunga Din:

## HISTAMINE

*By Carl A. Dragstedt*

You may talk of toxicosis,  
Migraine, shock, or halitosis—  
Two to one there'll be a heckler on the scene,  
Who'll report his observations,  
Mingled with imaginations,  
Placing all the blame on evil Histamine.

For it's 'mine, 'mine, 'mine,  
By your real name or your alias, ergamine,  
You give asthmatics wheezes,  
Pollinosis patients sneezes—  
You smooth-muscle stimulating Histamine.

Trauma, burns, and inflammation,  
Headache, pain, and constipation  
Show the fingerprints of some malicious fiend.  
And the one that gets accused  
Is that amine so abused—  
Beta iminazol ethylamine.



For it's 'mine, 'mine, 'mine,  
On you every research worker vents his spleen.  
Though obscure as yet, the fact is  
You're involved in anaphylaxis—  
You capillary poison—Histamine.

Since the days of Dale and Barger,  
His dossier gets longer, larger,  
And the files will show some chapters in between,  
Indicating he's suspected  
Of some items here neglected—  
That secretion stimulating Histamine.

For it's 'mine, 'mine, 'mine,  
You decarboxylated son of histidin.  
We've extracted you and weighed you,  
By the living gut assayed you,  
But we're yet to get your measure—Histamine.

1947

## THE ROLE PLAYED BY LEUCOCYTES AND PLATELETS IN ANAPHYLACTIC AND PEPTONE SHOCK

By M. Rocha e Silva

*Department of Biochemistry and Pharmacodynamics, Instituto Biológico, São Paulo, Brazil*

If one goes back to the earlier theories of anaphylaxis, the number of separate points of view, or schools, defending entirely independent ideas on the mechanism of this important phenomenon becomes quite impressive. We might distinguish among the different conceptions—the immunological, the hematological, and the physiological or pharmacological—according to the main principles involved and the personal interests and scientific outlook of their proponents. Today, it is rather trivial to say that the liberation of histamine plays a major role in the production of the symptoms and that other metabolites, such as adenosine, acetylcholine, a “slowly reacting substance” (S.R.S.), *etc.*, might also contribute to initiate or aggravate shock. There is also no question that heparin, as such, is released from the liver and that it could explain incoagulability of the blood, and that the opposite effect (*i.e.*, the decrease in clotting time as it has been observed in anaphylactic and peptone shock during the so-called negative phase) might depend upon activation or release of thromboplastic agents, among which one might include activation of a proteolytic enzyme of the trypsin type.

It is obvious, however, that this description does not include many other phenomena that occur in anaphylactic and peptone shock with at least an equal degree of frequency. I pass over the immunological point of view, since there is a general agreement that the whole anaphylactic reaction takes place as a consequence of a combination of the antigen with the antibody, this being a sort of trigger mechanism for the whole physiological process to develop. The fact that similar phenomena and symptoms can be obtained by injecting peptone led to the conclusion that the symptoms following combination of the antigen with the antibody are common to other forms of aggression to the cell and by no means represent the direct, toxic effect of the so-called antigen-antibody complex. Therefore, reconciliation of the physiological or pharmacological point of view with the immunological one is much easier than might appear by reading papers from either side.

But, now, let us consider the hematological point of view. First of all, I wish to mention the old French conception of the “colloidoclastic shock,” which implies a disturbance of the colloidal equilibrium of the blood, with consequent fall in leucocytes and platelets, as the main reason for the symptoms observed during anaphylactic and peptone shock. I never understood what point was involved in the idea of the disturbance of the colloidal equilibrium of the blood. There is no question, however, that something remained from this old conception. This refers to the fall in leucocytes and platelets as a characteristic feature of anaphylactic and peptone shock. More detailed studies have subsequently shown that a fall in platelets is one of the most constant indications of a state of sensitization when the antigen is injected, at least in certain species of animals. In 1924, Webb<sup>1</sup>

made an extensive study of the leucopenia which occurs in anaphylaxis in the dog, describing enormous accumulation of leucocytes inside of the liver parenchyme, as shown by histological methods. As concerns platelets, Kopeloff and associates<sup>2</sup> and Kinsell and associates<sup>3</sup> have shown that a decrease in platelets is definitely proportionate to the gravity of anaphylactic shock in monkeys and rabbits. At the same time, Kopeloff and Kinsell tried to correlate the rupture of platelets with the liberation of histamine, since rabbits' platelets are very rich in histamine.

The following scheme incorporates the theoretical explanation for their findings:

TABLE 1

INTERRELATIONS BETWEEN PLATELETS' DESTRUCTION AND HISTAMINE RELEASE IN THE RABBIT SUBMITTED TO ANAPHYLACTIC SHOCK\*

A. Sensitized animal + antigen → B. Platelet and W.B.C. destruction

Platelet destruction  $\xrightarrow{\text{is related to}}$  Changes in blood coagulability

Platelet and W.B.C. destruction  $\xrightarrow{\text{liberate}}$  Histamine

Histamine  $\xrightarrow{\text{produces}}$  Symptoms and some blood changes

\* According to Kinsell *et al.*<sup>3</sup>

This interesting correlation postulated by Kinsell and Kopeloff, between disintegration of platelets and leucocytes and the liberation of histamine, can only be valid in relation to the rabbit, since, as above stated, rabbits' platelets are a rich source of histamine. But, in the case of the dog, for instance, it is not so easy to explain liberation of histamine as a consequence of an explosive destruction of platelets, since those blood elements are very poor carriers of histamine in this species of animal. Moreover, it has been proved that most of the histamine which appears in the circulating blood during anaphylactic and peptone shock, in the dog, comes out of the liver.

The main attempt undertaken in the last years by myself and co-workers<sup>4-6</sup> has been to correlate the mechanism of liberation of histamine and other metabolites with activation of enzymes of the proteolytic type. It is a known fact that trypsin<sup>4</sup> and such other proteolytic enzymes as papain<sup>6</sup> and ficin<sup>6</sup> liberate histamine and, so far as trypsin is concerned, there is a demonstration that it also liberates heparin,<sup>7</sup> adenosin,<sup>8</sup> and the so-called "slowly reacting substance."<sup>8</sup> There is also ample evidence that, during anaphylactic and peptone shock, activation of proteolytic enzymes takes place,<sup>9-12</sup> and that this activation appears to be correlated with the very mechanism of production of those kinds of shock. This is what might be called the enzymatic point of view, and, according to evidence presented several times, it is not impossible to establish a connection between this enzymatic conception and the other pharmacological and hematological points of view previously mentioned. How it could be done now, with the evidence available, is an important point to be discussed in this paper.

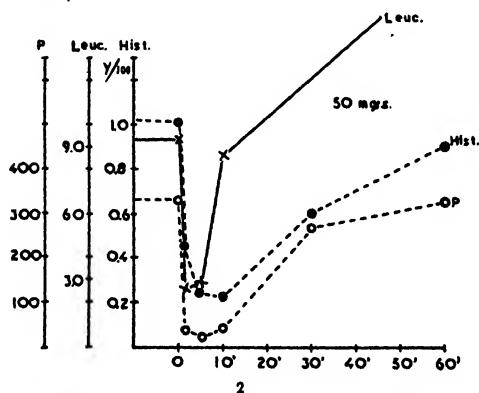
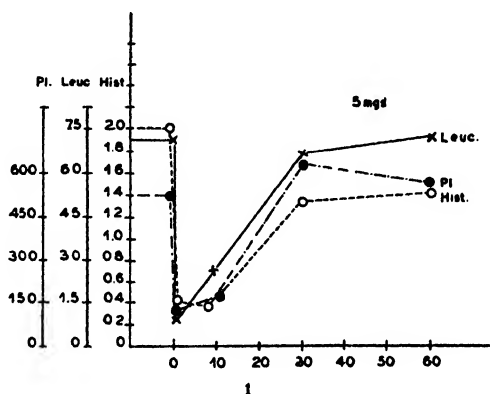
*Relationship Between Blood Histamine and White Blood Elements.* In 1937, on the basis of a fractionation of heparinized blood from different

species of animals, such as the goat, the calf, the dog, the rabbit, and the bullock, as well as from humans, Code<sup>13, 14</sup> concluded that 70 per cent of the blood histamine is bound to the white-cell layer, the so-called "buffy coat." As regards the dog, this connection between histamine and granulocytes, as established by Code in 1937, is probably correct. Also, in humans, it seems quite well established that the main source of histamine in the blood must be found in the leucocytes, although opinions diverge regarding the different lines of white blood cells to which histamine might be linked. . On the basis of observation of 5 cases of eosinophilia, Code and Macdonald<sup>15</sup> suggested that eosinophils are the main carriers of histamine in human blood. Randolph and Rackemann,<sup>16</sup> however, could not find any correlation between eosinophilia and the histamine content of the blood. In France, Parrot and Gabe<sup>17</sup> analyzed one case of great eosinophilia, in which a fractionation of the different elements of the blood showed that, although the platelets had the highest concentration of histamine, this base was found in larger amounts in the eosinophils. The neutrophils, according to the French workers, were very poor in histamine.

The most interesting case of the relationship between histamine and white blood elements, however, is presented by the rabbit. The blood of the rabbit is an important source of histamine, each cubic centimeter containing as much as 2 to 5 micrograms of the base. Barsoum and Gaddum<sup>18</sup> showed that the amount of histamine bound to the blood cells was 6 times greater than that of the plasma. Anrep and Barsoum<sup>19</sup> and Code and Ing<sup>20</sup> reported results showing that blood histamine in the rabbit is mainly bound to the leucocytes, since 70 per cent of the total blood histamine extractable from rabbit's blood might be found in the buffy coat. It is, however, a known fact that this buffy coat is usually heavily contaminated with platelets. Therefore, the fact that it is very rich in histamine would not prove that the histamine is mainly bound to leucocytes. Other methods have been used in this connection, and we can say that evidence is now in favor of this histamine's being bound to platelets in the blood of the rabbit. First, we have to mention the experiments of Minard,<sup>21</sup> in which he applied electrophoresis to extract the histamine from several portions of rabbit's blood and showed that histamine is concentrated in the platelets fraction of the blood in amazing quantities. The findings of Minard were extended by Zon, Ceder, and Crigler<sup>22</sup> in experiments in which antiplatelet serum was used to remove platelets from circulation.

The other method used in this connection consisted in following the values for blood histamine after the injection of polysaccharides extracted from certain parasites such as *Echinococcus granulosus* and *Ascaris lumbricoides* and also of glycogen from dog's liver. This method was extensively studied by Graña, Porto, and the author<sup>23, 24</sup> in a series of experiments to which I shall refer in detail. We had observed<sup>23</sup> that, when hydatid fluid (cysts of *Echinococcus granulosus*) was injected into rabbits, it did not produce a fall in blood pressure to contrast with its high toxicity, as observed when dogs are used as animals of experiment. The hematological changes, however, were strikingly similar to those found in rabbits submitted to anaphylactic

and peptone shock. Immediately after the injection of the hydatid fluid, leucocytes and platelets fell sharply in the circulating blood and simultaneously the blood histamine showed considerable decrease. When we started studying another helminth, the *Ascaris lumbricoides*, using the extracts prepared according to the technique described by Macheboeuf and Mandoul,<sup>25</sup> we found that extracts which were extremely toxic for guinea pigs and dogs failed to produce any symptom in the rabbit. Yet the same hematologica

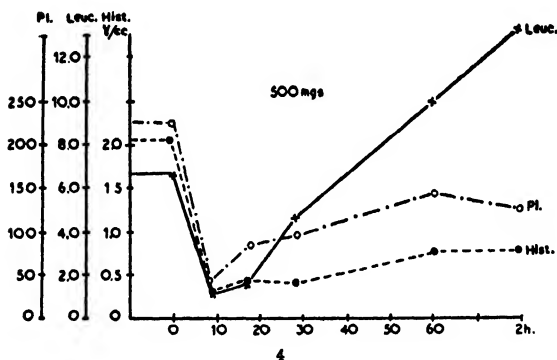
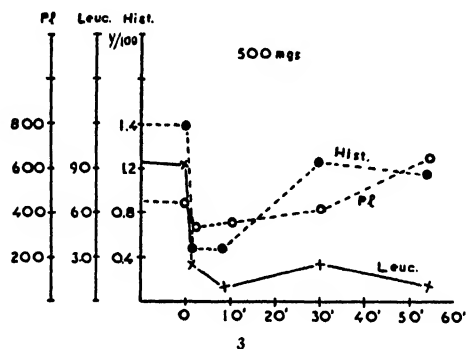


FIGURES 1, 2, 3, and 4. Variations of leucocytes, platelets, and blood histamine in normal rabbits receiving, intravenously (at 0), from 5 mg. to 500 mg. of liver glycogen per kg. of body weight. Note that the curves for platelets and histamine show a fairly good parallelism.

changes, namely leucopenia, thrombocytopenia, and fall in blood histamine, were also observed.<sup>24</sup> Fractionation of the hydatid fluid showed that a polysaccharide fraction was mainly responsible for this triad of hematological changes, analogous to those which are typical of anaphylactic and peptone shock. Similarly, fractionation of the *Ascaris* extracts employed has shown that glycogen-like material, which constitutes a substantial part of these extracts, was mainly responsible for the effect upon leucocytes, platelets and histamine when injected intravenously into rabbits.

These findings led us to try a purified glycogen, extracted from dog's liver. It was shown that glycogen produces a sharp fall in leucocytes when injected

into several species of animals.<sup>26</sup> We found that, at the same time, the platelets fall sharply and, also, that histamine is sharply decreased when very small doses of glycogen are injected into the veins of rabbits. FIGURES 1, 2, 3, and 4 show this effect of doses of glycogen ranging from 5 mg. to 500 mg. per kg. of body-weight. Compared with other substances, such as gelatin and agar, the ability of glycogen to produce drastic changes in the leucocytes and platelets and also in the blood histamine, is strikingly higher.<sup>27</sup> If one analyzes carefully the curves presented in FIGURES 1-4, one can see that, although in some cases the curves for leucocytes, platelets, and his-



tamine run a parallel course, in a few instances the leucocytes rose very sharply after an initial fall, while the curves for platelets and histamine werestilldepressed. In other cases, the opposite was observed, the leucocytes remaining low (FIGURE 4), while the histamine and platelets went up more swiftly. In all cases examined, it appeared quite evident that the histamine curves ran more parallel to the platelet curve than to the leucocyte curve. This is another excellent indication that histamine is mainly bound to platelets. In the experiments with hydatid fluid and *Ascaris* extracts, a similar parallelism was observed.<sup>23, 24</sup>

*Segregation of Leucocytes and Platelets in the Small Vessels of the Organs.* Another interesting observation in this series of experiments was the fact that, by injecting small amounts of glycogen, we could increase conspicuously

the histamine content of an organ, such as the lung. The experiments were done on rabbits in which thoracic aorta and inferior vena cava were clamped just above the diaphragm. In this "thoracic rabbit," if one injects glycogen intravenously, taking pieces of the lung before and after the injection, a considerable increase of the lung histamine, up to 7 times the previous quantity, could be observed (FIGURE 5). I call attention to this possibility of modifying the actual amount of histamine bound to the tissues by simply

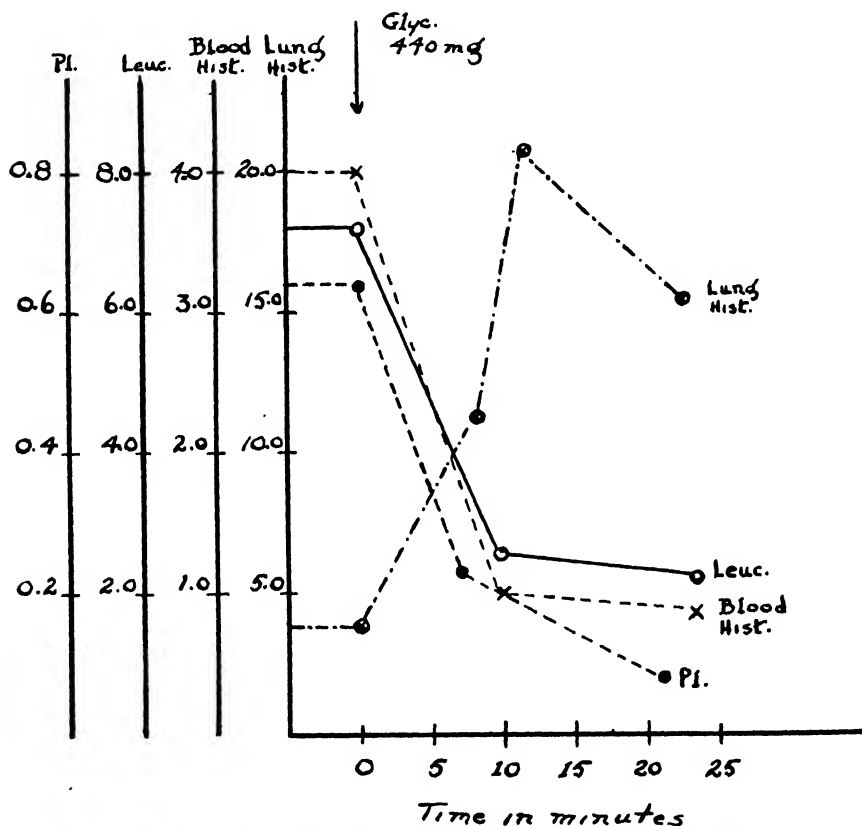


FIGURE 5. Variations of leucocytes, platelets, blood histamine, and lung histamine, after the injection of 440 mg. of liver glycogen per kg. of body weight, in a "thoracic rabbit" (inferior vena cava and thoracic aorta clamped just above the diaphragm).

changing the distribution of blood cells, especially leucocytes and platelets. Under the influence of glycogen, these elements are agglutinated and the agglomerates are captured by the lungs, forming clumps. It is obvious that an accumulation of elements which are very rich in histamine, inside an organ parenchyme, would lead to an increase of the actual amounts of histamine extractable from that organ.

I wish to emphasize that neither glycogen, hydatid fluid, nor *Ascaris* extracts produce any toxic effect on the rabbit. Therefore, a simple removal of the white blood elements from circulation, in the form of agglomerates or

clumps, is entirely incapable of producing any symptom of shock. Those polysaccharides are devoid of the capacity of liberating histamine from the blood cells when added *in vitro* to samples of rabbit's blood.<sup>23, 24</sup> In similar conditions, as we shall see later, the antigen or peptone produces a release of histamine. This can be shown by estimating plasma histamine with and without the addition of antigen and peptone. Another difference can be found in the fact that the aggregates of leucocytes and platelets, as produced by glycogen, remain intact inside of the capillaries of the lung or the liver. This can be shown by using a very simple technique to see the clumps of white blood elements inside of the organs. Since this technique constitutes an important source of information for the role played by leucocytes and platelets in anaphylactic and peptone shock, I shall describe it in detail.

It is evident that simple counting of leucocytes and platelets in the circulating blood will not give very much information on the real fate of those blood elements. By simply counting, we cannot decide whether these blood elements are only segregated or actually destroyed. To decide whether there is or is not destruction of platelets after they are agglutinated, we had to devise a technique to see these elements inside of the organs themselves. After several trials, we could obtain consistent results simply by cutting a small fragment of the organ within a 3.8 per cent solution of sodium citrate. An angle of the organ was dipped into the anticoagulant solution contained in a shallow dish and a small piece cut with sharp scissors beneath the surface of the liquid. The pieces were again cut into smaller fragments, and smears or impressions were made over slides and stained with Giemsa or May-Grünwald. When the pieces were taken from normal organs, a fairly uniform distribution of the platelets could be seen in the smears. If glycogen, for instance, is injected into a "thoracic rabbit," and smears of the lung made as described, enormous aggregates of platelets and leucocytes are seen in the slides. Those aggregates, formed after the injection of glycogen or any of the aforementioned polysaccharides, are stable, contrasting with the explosive destruction of platelets seen after the injection of peptone or the antigen, as will be referred to later.

To finish this subject, I should like to give a few indications of the possible mechanism by which glycogen might work in agglutinating platelets and leucocytes. There is no question that the blood elements become more sticky after the injection of glycogen. This fact has been shown by Essex and Graña,<sup>25</sup> using a transparent chamber adapted to the rabbit's ear. It is also apparent that glycogen does not work simply by its colloidal properties, since other colloids, like gelatine and agar-agar, are extremely less active in removing leucocytes and platelets from circulation than liver glycogen.<sup>27</sup> In this connection, it is interesting to recall some old experiments by Chambers and Grand,<sup>29</sup> in which they found that glycogen displays an extremely potent chemotactic effect upon leucocytes. This observation was confirmed and extended by Delaunay.<sup>30</sup> Although a further investigation is necessary to clarify the mechanism by which such minute amounts of glycogen produce a fall in platelets and leucocytes, it is unquestionable that glycogen might constitute an important tool as a reagent to remove



leucocytes and platelets momentarily from circulating blood. This technique was applied, in a few instances, to study the role played by leucocytes and platelets in those kinds of shock, as will be mentioned later on; but it is necessary to use the information obtained by this procedure with caution, since leucocytes and platelets are not removed from the body, but simply segregated in several unspecified structures. Therefore, the animal cannot be compared with a deplatelized or agranulocytic animal for all purposes. Some information, however, could be obtained simply by removing these blood elements from circulating blood.

*Anaphylaxis in the Rabbit.* The most interesting aspect of the relationship between blood elements and blood histamine can be studied as regards anaphylactic and peptone shock in the rabbit. In 1938, Abell and Schenck<sup>31</sup> observed the behavior of arterioles and capillaries, using the transparent moat chamber in the rabbit ears. After the injection of the antigen into sensitized animals, they observed arteriolar contraction with obliteration of the lumen, increased adherence of leucocytes to the endothelium of the blood vessels, and leucocytic migration through the walls of the capillaries and venules. Particularly interesting was the fact that leucocytes become sticky, adhering to form clumps or emboli and producing, in certain cases, stoppage of the circulation in the small vessels. Endothelial destruction with blood extravasation was seen in the most severe reactions.

That this clumping of leucocytes is of interest in explaining some of the peculiarities of the anaphylactic crisis in the rabbit, particularly the leucopenia, was shown by Dragstedt and associates<sup>32, 33</sup> in experiments of lung perfusion of a normal rabbit with heparinized blood, to which a mixture in optimal proportions of the antigen with the antibody was added. Leucocyte counts were made before and after the addition of the shock-causing mixture. There was a marked reduction in the leucocyte counts, amounting to approximately 50 per cent of the previous values, immediately after the first passage of the blood containing the antigen-antibody mixture. The conclusion to be taken from these experiments is that the lungs, or possibly other organs luxuriantly provided with capillaries and small vessels, may act as filters for the leucocytes and possibly also for the platelets, which become sticky after the injection of the shocking mixture. Since the lung constitutes the organ of shock in the rabbit, we can understand the significance of this plugging of the small vessels of the lung with microthrombi or emboli formed by agglutinated blood elements for the aggravation of the occlusion of the pulmonary artery bed.

There is, however, another aspect of this problem that must be analyzed here. The white cells of the rabbit (leucocytes and platelets) are the main bearers of blood histamine. Rose and Weil<sup>34</sup> and Rose<sup>35</sup> have shown that blood histamine sharply decreases in anaphylactic and peptone shock in the rabbit, and Goetzel and Dragstedt<sup>36</sup> have shown that a similar phenomenon takes place when peptone is injected intravenously in this kind of animal. Part of this decrease of blood histamine can be accounted for by the mechanical removal of white blood cells (leucocytes and platelets) from the circulation. It is conceivable that, if the main carriers of histamine in

rabbits' blood, namely leucocytes and platelets, are retained in some fixed structures, such as the capillaries and small vessels of the lung, the total histamine content of the blood must drop in the same way as in the experiments described with glycogen and other polysaccharides.

There is, however, a substantial difference between the experiments performed with glycogen and those in which the antigen or peptone is injected or added *in vitro* to samples of rabbits' blood. The difference consists in the more or less rapid diffusion of the histamine from cells to plasma, as shown first by Katz<sup>37</sup> and confirmed by others.<sup>32, 33, 38</sup> Rabbits sensitized to egg albumin were bled by cardiac puncture and the blood heparinized and submitted *in vitro* to the contact with the antigen. In all instances, the plasma of the blood, which had undergone this *in vitro* "shock," showed marked increase in the amount of histamine, indicating an ability of histamine to diffuse from cells to the surrounding plasma as a consequence of the presence of the antigen.

Dragstedt, Ramirez, and Lawton<sup>32</sup> confirmed these findings of Katz and calculated from *in vitro* experiments that the amount of histamine which could be liberated *in vivo* from rabbits' blood-cells would be about 0.1 to 0.3 mg. per kilogram of body weight. This amount is not enough to produce death in a rabbit when injected intravenously, but we must consider that anaphylactic shock in the rabbit very seldom follows a very severe course and that the histamine is probably liberated from the clumps of leucocytes and platelets formed in the intimacy of the capillaries and small vessels. Therefore, even minimal amounts of histamine might produce considerable pharmacological effects. It is logical, however, to assume that, besides the histamine which is liberated from the white cells, the mechanical plugging of capillaries with microthrombi formed by agglutinated blood elements might constitute an aggravating factor. Evidence for this interpretation was recently afforded,<sup>24</sup> by injecting large doses of glycogen in sensitized rabbits before the injection of the antigen. Leucocytes, platelets, and blood histamine were drastically reduced by the glycogen injection and the animal became refractory to further injections of the antigen. It appears quite clear that the previous injections of glycogen would disperse leucocytes and platelets all over the body, without allocating them to any specified vital structure, although the antigen or peptone would allocate them predominately to the capillaries and small vessels of the lungs, where the main events of anaphylactic shock take place.

At this point, I would like to formulate a set of general statements: (1) the release of histamine is connected with some kind of breakdown of the white blood elements; (2) the clumping of the white blood elements is a constant event during anaphylactic and peptone shock; (3) histamine might constitute a localizing factor for the capture of leucocytes and platelets, which have already begun to clump in the peripheral circulating blood; (4) obstruction of the small vessels and capillaries might depend upon two main factors, one chemical, probably histamine, and the other mechanical, the agglutinated white blood elements. In this explanation, the characteristic pharmacological effect of histamine, *i.e.*, the constriction of the pulmonary

artery branches in the rabbit would imprint its main pattern on the anaphylactic reaction by localizing the clumped elements to the capillaries of the organ of shock, the lung.

*Anaphylaxis in the Dog.* In 1924, Webb<sup>1</sup> made an extensive study of the leucopenia which occurs in sensitized dogs as a consequence of the injection of the antigen. Leucocytes were found in enormous quantities, especially in the liver. More recent studies dealt with the counting not only of leucocytes in the circulating blood but also of platelets.<sup>39, 40, 12</sup> There is no question but that the fall in platelets and leucocytes follows the same trend of the other characteristics of anaphylactic and peptone shock, but a strict parallelism could not be found so far as anaphylactic shock is concerned. It is a known fact that sometimes the platelets and also the leucocytes decrease sharply in circulating blood, after the injection of the antigen, without very much impairment in the blood pressure.<sup>39, 40</sup> This would prove that a fall in leucocytes and platelets is not enough to produce shock. In the case of peptone shock, however, the parallelism between the decrease in platelets in circulating blood and the fall in blood pressure is quite striking, as are the other characteristics of the shock, such as increase in blood histamine and heparin.<sup>12</sup>

There is, however, another point that has been overlooked by previous workers, namely destruction of platelets. Lysis or disintegration of these blood elements cannot be shown simply by counting them in peripheral blood, since they might be retained by some organ structures. Furthermore, disintegration occurs after the injection of glycogen. It is obviously necessary to see whether those clumps formed inside certain organs are or are not destroyed or disintegrated. We have been able to demonstrate this by using the technique, previously described, of making smears of pieces of certain organs for microscopic observation (FIGURE 6, A). In the intact dog, if one injects the antigen or peptone and takes a fragment of the liver immediately after the injection, one can see (FIGURE 6, B) enormous aggregates of platelets and also of leucocytes, scattered all over the slides. If the fragment is taken a few minutes later, a gigantic destruction of these clumps begins to occur, sometimes taking the course of an explosive lysis or disintegration of platelets. In their places, we can see, after a short while (FIGURE 6, C), only ghosts of the previous clumps, or a dust-like material, staining very well by Giemsa or May-Grünwald and unquestionably identifiable with remains or debris of platelets. By this direct method, we can therefore show that platelets are really disintegrated during shock.

As we saw in the case of the rabbit, it has been possible to establish a certain relationship between breakdown of platelets and the release of histamine that takes place when the antigen or peptone is put into contact with samples of rabbits' blood. Conditions in the dog are entirely different, since the histamine which appears in circulating blood during shock comes mainly from the liver. Is it possible, however, to establish a similar relationship, even in the case of the dog?

The participation of the liver in the production of anaphylactic and peptone shock has been suspected since early times. Manwaring<sup>41</sup> was unable



FIGURE 6

A. Smear of a piece of normal dog liver, taken directly into a 3.8% solution of sodium citrate. Stained by May-Grünwald. Note the homogeneous distribution of platelets among red cells (450X enlargement).  
 B. Fragment of the liver of a dog submitted to shock by injection of the antigen from *Ascaris fimbrioides*. Enormous clumps of platelets are seen in the slides, made with a fragment of liver taken before 2 minutes after the injection of the shocking agent (450X enlargement).  
 C. Impression over slide of a piece of the liver of the same animal, 10 minutes after the injection of the *Ascaris* antigen. The clumps previously seen, are entirely disintegrated and in their place only remains of platelets or ghosts of clumps can be seen (450X enlargement).

to elicit anaphylactic shock after ligation of the aorta and inferior vena cava just above the diaphragm. Also, the injection of the antigen in a sensitized dog with an Eck fistula does not produce shock, as shown by Voegtlin and Bernheim<sup>42</sup> and Denecke.<sup>43</sup> The extreme stagnation of blood in the portal region was already described by Richet,<sup>44</sup> and shown by Weil<sup>45</sup> to be quantitatively enough to explain fall in blood pressure and death of the animal. More recently, Ojers, Holmes, and Dragstedt<sup>46</sup> showed that most of the histamine appearing in the circulating blood comes out of the liver, since histamine estimations of pieces of the liver taken before and after the injection show considerable decreases following the development of the shock. A similar discharge of histamine from dogs' liver in peptone shock was described by Holmes, Ojers, and Dragstedt.<sup>47</sup> That heparin is also liberated from the liver has been shown repeatedly.

In 1943, Graña and the author<sup>48</sup> decided to show by perfusion experiments the hepatic origin of this histamine that appears in the blood of the dog during anaphylactic shock. We started using Tyrode solution as vehicle for the perfusion. To our great astonishment, not a single  $\mu\text{g.}$  of histamine or heparin appeared in the perfusates after the injection of the antigen. Using peptone, we could detect a few micrograms of histamine (not more than 10 or 12), contrasting with the milligrams which are released in the intact animal, but no heparin could be detected.<sup>12</sup> Later on,<sup>49</sup> we decided to experiment by using citrated blood contained in paraffined receptacles. Under these conditions, some histamine and heparin were released, but not as much as can be released *in vivo*. Finally, in collaboration with Scroggie, Fidlar, and Jaques<sup>7</sup> we decided to exclude the anticoagulant from our experiments of liver perfusion, using blood kept in its natural conditions by the use of flasks covered with silicones. As shown by Jaques *et al.*,<sup>50</sup> the blood is kept unclotted for at least 2 hours, and even platelets are well preserved during the first half-hour after collecting the blood. By adding peptone to that blood, and pumping it through the liver, we have been able to detect enormous amounts of histamine and heparin, which were liberated in quite an explosive way during the first minutes of perfusion. As much as 8.5 mg. of histamine and more than 50 mg. of heparin could be liberated in one of the experiments of liver perfusion with silicone blood plus peptone.

These experiments provided the final proof that the blood contains a factor or factors which are necessary for the discharge of histamine and heparin from the liver of the dog during anaphylactic and peptone shock. Since the anticoagulant seems to constitute a disturbing factor, and the best results were obtained when the blood was kept as near as possible in its natural condition, we have to conclude that the most labile part of the blood, namely leucocytes and platelets, are probably involved in the production of this discharge of histamine and heparin. What was the fate of these blood elements after perfusion of the liver with citrated or silicone blood? In all those experiments, leucocytes and also platelets were counted before and after the passage of blood plus antigen or peptone through the isolated organ. Immediately after the injection of the antigen or peptone, leucocytes and platelets were instantaneously picked up and were found to be forming aggregates inside the organ parenchyme. If citrated blood is used for the

perfusion, those clumps of leucocytes and platelets are found well protected and intact at the end of the perfusion. Also, the amounts of histamine and heparin liberated under these conditions were rather small. If, however, silicone blood is used for the perfusion, leucocytes and platelets are likewise picked up by the organ, but the clumps formed are found to be completely disintegrated at the end of the experiment and enormous amounts of histamine and heparin can be detected in the perfusates. This parallelism between destruction of white blood elements and the discharge of histamine and heparin from the liver cells, which is so striking in the experiments with the isolated liver of the dog, can also be found *in vivo*, if fragments of liver are taken before and after the injection of the shocking agent.<sup>49</sup>

Other evidence pointing in the same direction was given by experiments in which liver glycogen was injected into the intact dog before the *Ascaris* extract. As previously mentioned, extracts prepared from *Ascaris lumbricoides*, according to the technique of Macheboeuf and Mandoul,<sup>25</sup> produce a profound shock in the dog that cannot be distinguished from anaphylactic shock.<sup>48, 49, 51</sup> Enormous amounts of histamine are liberated from the liver and can be estimated in the circulating blood. Simultaneously, a profound fall in platelets and leucocytes takes place in the circulating blood and, if pieces of the liver are taken for microscopic observation, enormous aggregates of platelets and leucocytes can be found in the piece taken immediately after the injection (FIGURE 6). If another piece is taken a few minutes after the injection, a considerable destruction of the formed clumps can be observed, amounting to a real "explosion" of platelets. Leucocytes also are shown to be heavily damaged. If, before the injection of the *Ascaris* extract, we inject a large dose of glycogen, 3 to 8 gm. in 10 to 20 cc. of saline, platelets disappear almost completely from the circulating blood and no shock occurs. If then, the *Ascaris* extract is injected, the effect is somewhat modified, as concerns the fall in blood pressure, since the shock is definitely mitigated.<sup>48</sup> What is striking, however, is the almost complete inhibition of the increase in histamine and heparin in the circulating blood. The interpretation given to this effect of a previous injection of glycogen was that a dispersion of the white blood elements would prevent any serious accumulation of the same inside the liver parenchyme, thus bringing about a situation unfavorable to the discharge of histamine and heparin.

We have evidence, therefore, that in the dog the discharge of histamine and heparin from the liver parenchyme depends upon a previous breakdown of the white blood elements: (1) the blood is important for the discharge, and the natural condition of the blood must be preserved in order to have a maximal liberation of histamine and heparin from the isolated liver; (2) disintegration of platelets and leucocytes is somewhat correlated with the intensity of the shock and also with the discharge of histamine and heparin from the liver; (3) a previous dispersion of the white blood elements, by the injection of high doses of glycogen, partially desensitizes the animal to shock and prevents liberation of histamine and heparin from the liver of the dog, kept *in situ*. To this indirect evidence, I might add the fact that the liver of the sensitized dog, when perfused with citrated or heparinized blood, picks up sharply the white blood elements, which are then retained inside of the

organ, forming enormous aggregates or clumps. It is logical to assume that these microthrombi might help in occluding the small vessels of the liver, already partially occluded by the action of minute amounts of histamine.

On the basis of these facts, one might draw the following scheme for the mechanism of anaphylactic and peptone shock in the dog: (1) immediately after the injection of the antigen or peptone, there occurs an initial constriction of the hepatic vessels, effective in transforming the capillaries of the liver into a filter for the agglutinated blood elements; (2) the leucocytes and platelets agglutinated in contact with the antigen will form microthrombi in the small vessels and capillaries of the liver; (3) after the latent period, the platelets will "explode," this being followed by the liberation of histamine and heparin.

*Activation of Proteolytic Enzymes during Anaphylactic and Peptone Shock.* By which mechanism are products derived from platelets or leucocytes concerned with the liberation of histamine and heparin from dog's liver? It is possible that leucocytes and platelets might contain an enzyme of the proteolytic type, the release of which, during shock, might bring about the liberation of those metabolites. We have now, however, several indications that the protease system of the blood itself might constitute the missing link in the chain of events leading to the release. This plasma protease has been described in the literature under the names of "plasma trypsin,"<sup>62</sup> "plasma trypsinase,"<sup>63</sup> "lytic factor,"<sup>64</sup> "plasmin,"<sup>65</sup> and, more recently, "fibrinolysin."<sup>66</sup> The difficulty in showing its activation during peptone and anaphylactic shock resided in the fact that the blood becomes incoagulable after shock, due to the discharge of heparin. Therefore, if no clot is formed, it is obvious that the fibrinolytic effect could not possibly be observed.

To overcome this difficulty, we have used the protamine test, as described by Jaques and Waters.<sup>67</sup> After addition of a suitable amount of protamine, the blood clots. If the samples are incubated at 39°, a more or less rapid redissolution of the coagulum can easily be observed, especially in the tubes containing the largest amounts of protamine. It appears quite clear that protamine disposes not only of the heparin released, but probably also of the inhibitor present by fixing it up as soon as its connection with the enzyme had been broken down during shock. Using this fibrinolytic protamine test,<sup>11, 12</sup> we have been able to correlate the intensity of the fibrinolytic effect to the degree of shock: fibrinolysis is maximal after a first injection of the shocking agent, moderate after the second injection of peptone, and null after a second injection of the antigen or a third injection of the peptone. All the other symptoms of the shock, such as fall in blood pressure and liberation of histamine and heparin from the liver, follow the same trend. This led us to the conclusion that the fibrinolytic effect is connected with the mechanism of production of those kinds of shock, and that desensitization might depend either on an exhaustion of the mechanism leading to fibrinolysis or on the discharge of any antifibrinolytic agent, which would block the first wave of fibrinolysis. Probably both mechanisms might be operative in normal conditions.

That this antifibrinolytic agent seems to come from the liver was suggested by experiments performed in the so-called "anterior animal" or

"thoracic dog." This kind of preparation, already used by Nolf,<sup>9, 10</sup> can be set up simply by clamping thoracic aorta and inferior vena cava, just above the diaphragm. In that case, the fibrinolytic effect is very strong and much more pronounced and lasting than that observed in the intact dog, when peptone is injected. After the injection of peptone in the thoracic animal, we can observe a strong fibrinolytic effect until the end of the experiment, and no heparin appears in the samples. Therefore, we can be sure that all the samples taken from a thoracic animal, after the injection of peptone, will fibrinolyse. In this preparation, there is no recovery after the development of the fibrinolytic activity. Using this preparation, we have been able to demonstrate that a previous injection of heparin can prevent the activation of the fibrinolytic enzyme. In the protamine test, heparin does not prevent the effect of the fully activated enzyme, since, when the blood is

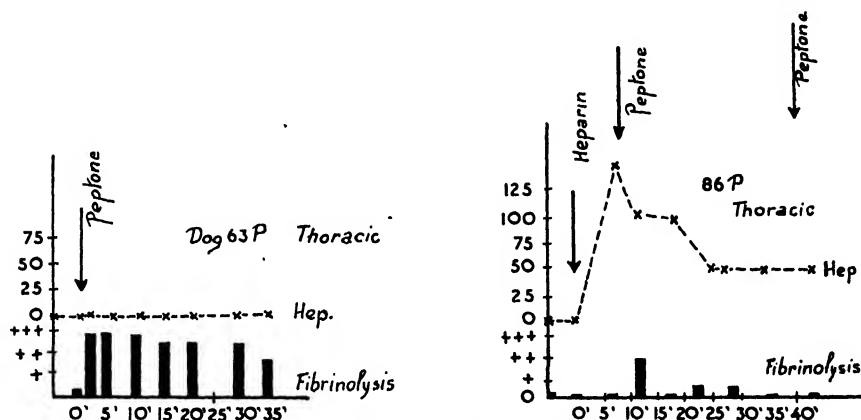


FIGURE 7. Left: Fibrinolytic effect developed in a "thoracic" dog, after the injection of 150 mg. of peptone (Bacto-peptone) per kg. of body weight. Right: Before the injection of peptone in a "thoracic" dog, 6 mg. of heparin per kg. of body weight were injected. Fibrinolysis was considerably reduced. In both experiments, the heparin curves indicate the amounts of protamine per 0.5 ml. of blood, that brings coagulation time to a minimum (+++ means fibrinolysis in less than 1 hour; ++, in less than 2 hours and +, in 3 hours or more).

collected in a syringe containing an excess of heparin and then distributed over the tubes of the protamine series, it will undergo fibrinolysis (as will the controls) without heparin. When heparin is injected before the peptone, however, it will sometimes entirely block the development of the fibrinolytic phenomenon in the thoracic preparation (FIGURES 7 and 8).<sup>88</sup>

The last point I wish to emphasize is concerned with the fate of platelets in this thoracic preparation. Immediately after the injection of peptone, the platelets and leucocytes disappear from circulation and are found forming aggregates in the intimacy of the lung parenchyme. In order to see these clumps, pieces of the lung were taken directly into a 3.8 per cent solution of sodium citrate, as described before. Immediately after the injection of peptone, we can detect a great number of clumps scattered all over the slides. These clumps are formed by a great number of platelets surrounding several leucocytes. If five or ten minutes are then allowed to elapse and another piece of the lung taken in citrate, the smears will show all those



clumps entirely disintegrated and destroyed. In their place, one can see only debris of platelets, formed by small granulations well stained by Giemsa and May-Grünwald. Only exceptionally, one can detect small but heavily damaged clumps of leucocytes and platelets. Also, the fibrinolytic activity of the blood is fully developed in this case.

Now, if heparin is injected before peptone, the platelets will clump as before, but the clumps can be seen to be well formed and intact long after the injection of peptone. I think that this effect of heparin, protecting the clumps of platelets against destruction and, at the same time, preventing activation of the fibrinolytic enzyme of the blood, constitutes good evidence that products derived from platelets might be concerned with the activation of this fibrinolytic enzyme of the blood during anaphylactic and peptone shock.<sup>58</sup>

It is a known fact that this plasma protease is normally present in the blood in an inactive form<sup>55, 59</sup> and that, at the same time, there is an in-

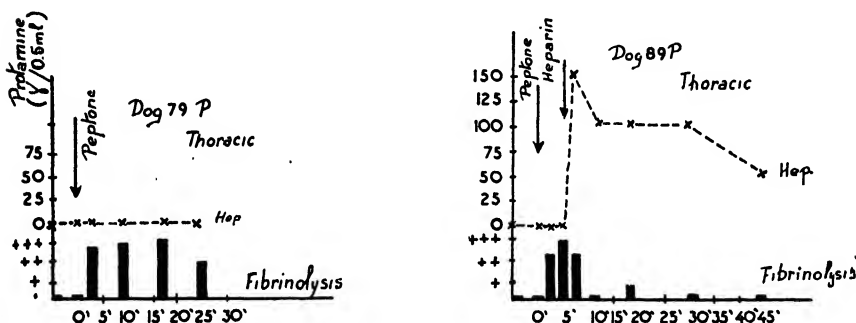


FIGURE 8. *Left:* Fibrinolytic effect in a "thoracic" dog after the injection of 150 mg. of peptone (Bactopeptone) per kg. of body weight. *Right:* Before the injection of peptone in a "thoracic" dog, 6 mg. of heparin per kg. were injected; activation of the fibrinolytic power was stopped in a very early stage. (Signs and methods the same as in FIGURE 7.)

hibitor preventing it from becoming apparent.<sup>52</sup> Since this enzyme appears to be spontaneously activated under certain morbid conditions, as in traumatic shock,<sup>60</sup> it is obvious that there must be an endogenous activator that might also be mobilized in cases of anaphylactic and peptone shock. As previously shown, there are indications that platelets might provide for this activator. As concerns the mechanism of activation of this enzyme, it might depend not only on a decrease of the inhibitor, allowing the spontaneous activation of the enzyme to take place, but also on the actual activation of the enzyme precursor (tryptogen,<sup>53</sup> plasminogen,<sup>55</sup> or pro-fibrinolysin,<sup>56</sup> according to the name given to the active enzyme) by a kinase, since it has been shown<sup>59</sup> that activation can take place without removal of the inhibitor. This last possibility seems to be the case in anaphylactic and peptone shock, since the samples of blood taken after shock, showing strong fibrinolytic effect when added immediately to the protamine series, will return to normal, after a few hours at room temperature, if no protamine is added at the beginning. This experiment shows that the enzyme recom-

bines with the inhibitor still present in excess in the blood samples and that, if the blood is kept unclotted (without addition of protamine) by the presence of the released heparin, no fibrinogenolysis occurs, since, if protamine is added a few hours later, the samples will clot but no lysis will occur.

*Probable Chain of Events Leading to Histamine Release.* Recently,<sup>49</sup> we have drawn up a scheme which attempts to correlate all the facts here de-

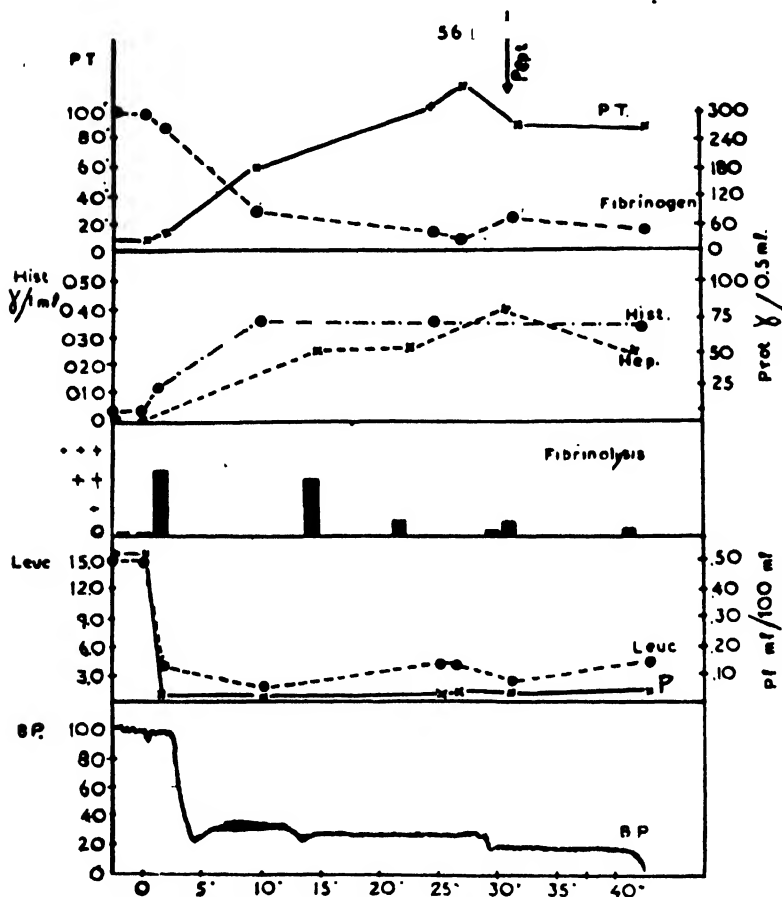


FIGURE 9. Anaphylactic shock in a dog sensitized to horse serum. At 0, 1 ml. of horse serum per kg. of body weight was injected intravenously. The stratified graph shows all the variables of the shock, from down up: fall in blood pressure (B.P.); fall in leucocytes (Leuc.) and platelets (P); the fibrinolytic effect as shown by the "fibrinolytic protamine test," increase in histamine and heparin in the peripheral blood; decrease in the "estimated" fibrinogen and increase in prothrombin time (P.T.). The two last changes are mainly due to increase in blood heparin.

scribed in a chain of enzymatic and pharmacological reactions (FIGURES 9 and 10). Under the present circumstances, this scheme does not pretend to be more than a simple working hypothesis, the fate of which will depend upon future investigations. Up to now, however, all the facts described in the literature appear to fit quite well into its lines. It is quite probable

that a similar chain of events might work in the case of the rabbit, with the difference that the main organ of shock in the rabbit is the lung, but, since platelets are a rich source for histamine in this species of animal, it is obvious that a simple breakdown of platelets might liberate enough histamine to explain shock in the rabbit. In the guinea pig, conditions seem to be even simpler, since the direct contact of the antigen with the cells of the organ of shock (the lung) is enough to produce the discharge of histamine, as shown in the classical experiments of Bartosch, Feldberg, and Nagel<sup>61</sup> and in those later on described by Ungar and Parrot<sup>62</sup> and Schild<sup>63</sup> with isolated pieces of several organs. It seems, however, that here an enzymatic mechanism also appears to be operative, since Ungar<sup>64</sup> quite recently showed activation of a fibrinolytic enzyme when organs of the guinea pig are put into contact with the antigen or with peptone.

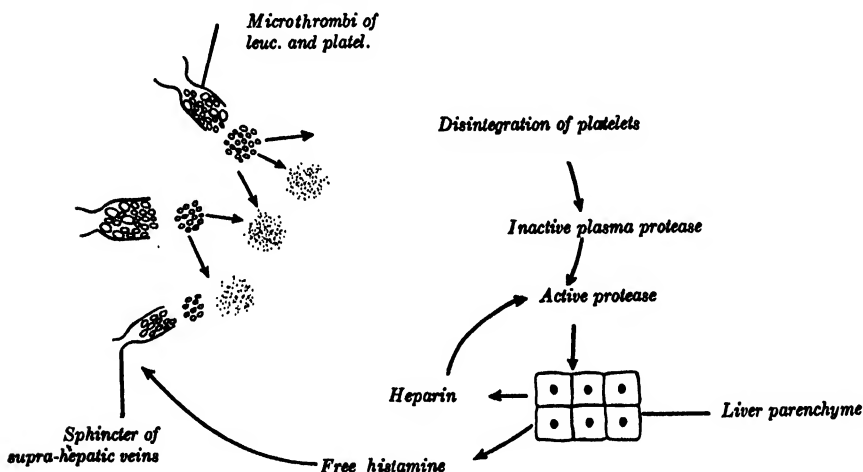


FIGURE 10. Probable chain of events leading to the discharge of histamine and heparin from dog's liver, in anaphylactic and peptone shock (according to Rocha e Silva, Porto, and Andrade<sup>19</sup>).

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### Discussion

E. FIDLAR (*Department of Physiology, University of Toronto, Toronto, Canada*): If platelet disintegration yields an activator for plasma trypsin, why is the latter not active in those cases of experimental purpura where platelets may suddenly be destroyed? Perhaps the trypsin is active; I do not know.

M. ROCHA E SILVA: This is a very interesting question. It opens up the problem of the relationship between the mechanism of purpura and histamine release in anaphylactic and peptone shock. It is a known fact that thrombocytopenic purpura is a common symptom of hypersensitivity, as, for in-

stance, in cases of drug allergy. The mechanism of production of the hemorrhagic spot is still unknown, but some time ago, in collaboration with Dr. Martins, we studied the mechanism of production of purpura by injection of antiplatelet serum. There is no question but that, under the influence of A.P.S. applied locally to the skin of the dog, rabbit, or rat, platelets clump, forming microthrombi or emboli which disintegrate just before the hemorrhage sets in. Therefore, we might assume that the cause for the hemorrhage in thrombocytopenic purpura is not the absence of platelets, as generally said, but that products of disintegration of platelets liberate or activate a hemorrhagic agent that might be a proteolytic enzyme of the trypsin type, since trypsin itself, always used as a model enzyme to minimize many of the anaphylactic reactions, is strongly hemorrhagic. Moreover, in the preparation described as the "thoracic dog," we have found that injection of a potent antiplatelet serum brings about a slight but detectable activation of the fibrinolytic power of the blood, in a way parallel to the disintegration of the clumps of platelets which form inside of the lung parenchyme.

J. ALEXANDER (*New York, N. Y.*): It is not clear to me as to just how histamine appears and how it acts to cause sudden contraction of certain non-striated muscles.

Since Dr. Rocha e Silva reports that he has observed the clumping of blood platelets and leucocytes in the dog (microthrombi), I trust that the notion of a possible connection between even nonpersistent flocculates and the development of clinical symptoms can be discussed. The experiments of Dr. Auguste Lumière (*Le Probleme de l'Anaphylaxie*, G. Doin, Paris, 1924) may be recalled in this connection, even though I have been informed that they led to what is now called "anaphylactoid" shock (due to arteriole compression following capillary blockage) rather than to true anaphylactic shock (due to contraction of arteriole musculature).

Lumière found that freshly precipitated barium sulfate, a chemically inert substance, caused no symptoms when intravenously injected into dogs. However, as the precipitate was aged and its particles, as observed microscopically, grew progressively larger, symptoms of increasing severity appeared, leading to violent shock and death, with aggregates of sufficient size. Since the capillary systems of various organs in various animals differ greatly in their vulnerability to blockage by barium sulfate, the clinical consequences vary with the animal used.

I therefore ask: Can flocculates, even if not persistent and not large or firm enough to block capillaries, nevertheless interfere temporarily and locally with supply and elimination, so that cells or other constituents of affected organs or tissues will face changed milieu conditions and react abnormally, one consequence being the release of histamine? Slight interference with blood circulation in the brain may have wide nervous and muscular consequences.

M. L. TAINTER (*Sterling-Winthrop Research Institute, Rensselaer, N. Y.*): The phenomena referred to may be produced by intravenous injection of substances that agglutinate red cells by chemical, physical, or immunological mechanisms. The agglutinated masses block capillaries, especially in the

lung bed. These changes have been extensively studied by Hanzlik and Karsner, under the name of "anaphylactoid phenomena." They grossly resemble anaphylactic shock. The mechanism appears to depend on a mechanical effect of compression of the smaller bronchioles by distension of adjacent capillaries.

M. ROCHA E SILVA: The experiments of Lumière are typical of the kind of explanation that might be classified under the general name of physical theories. They are now well buried under the mass of chemical and pharmacological data presented in the literature since then. I think that Dr. Alexander overemphasized the importance I wished to attach to the mechanical clumping of leucocytes and platelets with the consequent formation of microthrombi. I took for granted that the most important part of the phenomenon should be attributed to disintegration of white blood elements. Simply blockading the circulation of the liver leads to questionable, if any, release of histamine and heparin. It would, by no means, be enough to explain the explosive discharge that sometimes occurs in a few minutes, as shown in the experiments of perfusion of the isolated liver of the dog with peptone. I tried to make clear that products derived from platelets, and in smaller scale from leucocytes, might initiate a chain of enzymatic reactions, leading to the release of metabolites. It is true that I described<sup>48</sup> an instance in the dog, that I tentatively called "mechanical shock," in which the animal appeared to die by a mechanical stoppage of the circulation of the liver without very much discharge of histamine and heparin into the circulating blood. I should not like, however, to give too much emphasis to the "mechanical" nature of this kind of shock.

C. F. CODE (*The Mayo Clinic, Rochester, Minnesota*): Since MacIntosh and Paton have recently shown that simple substances like the tripanocidal diamidines, and the even simpler straight-chain diamines, diamidines, and diguanidines, release histamine from tissues, should not the possibility be considered that a simple substance or a group of simple substances similar in action to these compounds may be the chemical mediator for the release of histamine in anaphylactic and peptone shock, rather than the chain of enzymatic reactions described by Dr. Rocha e Silva?

M. ROCHA E SILVA: The question raised by Dr. Code implies that I am trying to complicate unnecessarily the picture of anaphylactic and peptone shock by bringing activation of a proteolytic enzyme into the picture to account for the liberation of histamine, heparin, and other metabolites. The reasons for bringing this in are presented in numerous publications from my laboratory, and they have been discussed only partially here. There is no question that activation of proteolytic enzymes takes place during anaphylactic and peptone shock and that it might explain a great portion of the symptoms observed, especially the hemorrhagic phenomena (in the intestinal tract of the dog and those that are characteristic of the Arthus' reaction in the rabbit), and necrosis, since histamine is unable to explain these effects. Proteolytic enzymes by themselves, as it was shown for pancreatic trypsin, display a number of pharmacological properties which are closely akin to the symptoms of anaphylactic and peptone shock. Besides that, the fall in platelets and leucocytes, with their consequent disintegration, are

# THE DETOXIFICATION OF HISTAMINE

By E. T. Waters\*

*Department of Physiology, University of Toronto, Toronto, Canada*

When considering the mode of removal of histamine from tissues, one might, with considerable justification, substitute the term "inactivation" for the term "detoxification." If further evidence is forthcoming to substantiate the present suggestive evidence that histamine fulfills various physiological roles, then the term "inactivation," applied to what would then be a normal functioning metabolite, would be less open to objection than the term "detoxification." Indeed, as applied to other states, where the quantity of free histamine in the tissues is unusually large, the connotation of the term "inactivation" is less open to objection than the rather popular term "detoxification."

It has been demonstrated many times that, following the parenteral injection of histamine into an animal such as the dog, rabbit, or rat, there is a rather rapid removal of the histamine from the circulating blood and, provided that the dose administered is not too large, the symptoms of histamine shock are ameliorated and finally disappear. Similarly, experiments have been carried out in which various isolated tissues or organs have been perfused with blood containing added histamine. Thus, Best and McHenry<sup>1</sup> and Steggerda, Essex, and Mann<sup>2</sup> showed, in perfusion experiments, that the dog's kidney was the most effective in the destruction of histamine. The liver was less effective, and, in perfusions of the small intestine or of the hind limb, little inactivation occurred. It is with the mechanisms or possible mechanisms of this inactivation of histamine by the tissues that we shall now deal.

It may be that histamine combines, by a simple chemical linkage, with the proteins of cell structures. Thus, it is removed from its sphere of action and is thereby rendered inactive, at least temporarily. This is suggested in many experimental findings and is discussed by some authors.<sup>3, 4, 5</sup> One might also refer to the report of Anrep and his associates<sup>6</sup> that, when histamine is added to a sample of blood, it is, in part, quickly taken up by the red cells and is thereby rendered physiologically inactive. If such blood is extracted for its histamine content, the added histamine may be recovered. We shall return to this aspect of histamine inactivation later.

Inactivation may also occur by conjugation, as shown by the presence in urine of an inactive form of histamine, readily activated by hydrolysis. Inactivation may also occur in tissues, as the result of the activity of the enzyme, histaminase, or, as some would prefer to call it, diamine oxidase.

From time to time, histamine has been detected, usually in small amounts, in the urine of man and other animals. The most thorough study of the presence of histamine in urine has been made by Anrep and his co-workers. They<sup>7</sup> reported in 1944 that, if urine was hydrolyzed with hydrochloric acid, while it frequently showed no histamine activity before hy-

\* Present address: Institute of Physiology, Cardiff, Wales.

hydrolysis, after hydrolysis there was often unmistakable evidence of histamine. They showed that the histamine is excreted in a conjugated and inactive form, which is freely diffusible and from which the active base can be liberated by hydrolysis, either by acid or by alkali. This increase in the histamine-like activity of urine after hydrolysis might have been due either to the destruction of some antagonizing substances interfering with the contraction of the guinea-pig ileum, used in the test, or to a release of the active principle from a conjugated or inactive form. They excluded the first possibility by showing that, when histamine is added even in traces to the nonhydrolyzed extracts, it can be estimated quantitatively on any of the usual test objects. Conjugated histamine, like the free base, is soluble in water and in alcohol and is readily adsorbed from urine by charcoal (B.D.H. decolorizing charcoal), from which it can be released by repeated elution with acidulated alcohol (0.3 N HCl in 95 per cent alcohol). In testing the action of histaminase, they found that the enzyme is entirely inactive in the presence of urine and that this inhibitory influence is retained by the extracts of urine even after acid hydrolysis. The inhibition becomes negligible on dilution. Even so, histaminase does not act on conjugated histamine.

There was no appreciable difference between the urines of a large number of different species of herbivora examined. The extreme variation was 0.02–0.2  $\gamma$ /cc. urine, calculated as histamine acid phosphate. There was no marked variation in urine collected from typical carnivora, but the histamine equivalent of all urines was high, 10 to 40  $\gamma$ /cc.

Animals on a varied diet gave considerable variations in the values of urine histamine. Thus, the white rat showed variations between 0.1 and 10.2  $\gamma$ /cc. and the dog varied between 0.2 and 45.0  $\gamma$ /cc. of urine. There appeared to be no relationship between the histamine equivalents of blood and urine, but it appears that the excretion of histamine into the urine depends upon the nature of the diet. In herbivora, histamine is chiefly in the free form, while in carnivora the histamine is completely conjugated. In the urine of the rat and of man, both forms are found.

When histamine was injected subcutaneously into dogs, there was no change in the amount of conjugated histamine in the urine and there appeared only a slight amount of free histamine, which is not usually present. The longer the period of anuria, following the injection of histamine, the smaller was the excretion of histamine. In any case, the bulk of the histamine was destroyed within the body. Histamine is present in meat in the free form, and the increase in histamine after the feeding of meat is partly due to the preformed histamine in the meat, but apparently not wholly so. The feeding of large amounts of histamine (up to  $\frac{1}{2}$  g.) to dogs results in an increase in conjugated histamine, the increase being about 3 to 5 per cent of the free histamine fed. From the dog, they have obtained, in 24 hours, over 30 mg. of histamine (calculated as acid phosphate), and sometimes the concentration is as high as 100  $\gamma$ /cc. of urine. The conjugated histamine has similarly been isolated from human urine. Subcutaneously injected conjugated histamine is almost quantitatively excreted. The oral administration of conjugated histamine leads to a



rapid and much greater excretion of it in the urine than when histamine acid phosphate is fed. Between 50 and 60 per cent of the amount fed is excreted in the unchanged form.

This conjugated or combined histamine is freely dialysable and should be distinguished from the combined form of histamine, presumably with protein, which is nondialysable. Very little is known of the chemical nature of this conjugated form, nor is the site of its formation known, whether kidney, liver, or intestine. From what has been said, it will be appreciated that the elimination of histamine, in either the free or the combined form, into the urine plays only a small part in the removal of histamine from the body.

In 1929, Best<sup>8</sup> reported that naturally occurring and added histamine disappears during the autolysis of minced lung of the horse and cow. This work was extended and, in the following year, Best and McHenry<sup>1</sup> suggested that the substance or system which produces a change in structure responsible for the loss of biological activity of histamine be designated "histaminase" and the reaction be referred to as the "histamine-histaminase" reaction. Later, in 1938, Zeller<sup>9</sup> showed that a hog-kidney preparation not only oxidized histamine, but oxidatively deaminated a variety of other diamines, such as ethylene diamine, trimethyl diamine, putrescine, cadaverine, and agmatine, and also<sup>10</sup> a variety of substituted diamines, *e.g.*, spermine and spermidine. Zeller therefore recommended that the enzyme be called "diamine oxidase" rather than histaminase.

Diamine oxidase, or histaminase, is widely distributed in the animal kingdom. It has been found in cold-blooded animals, in birds, and in mammals. Recently, Roulet and Zeller<sup>11</sup> have reported a diamine oxidase from an acid-fast bacillus, with a very low activity towards histamine. It has been shown<sup>12-14</sup> that most mammalian kidneys, with the exception of those of the rodents, are rich sources of the enzyme. The cortex usually contains more activity than the medulla, except in man, where the enzyme content is approximately the same in cortex as in medulla. In the newborn child, the medulla has a much higher content of diamine oxidase than has the cortex. In most mammals, with the exception of the rodents, the kidney contains more histaminase than the liver, whereas, in birds, this relationship is reversed. The intestinal mucosa of practically all warm-blooded animals contains histaminase.

It was shown by Best and McHenry<sup>1</sup> that oxygen is necessary for the inactivation of histamine by histaminase. Several groups of workers since have attempted to correlate the oxygen consumption with the amount of histamine inactivated. Laskowski<sup>15</sup> clearly demonstrated that the rate of oxygen uptake varied with the purity of his preparation of histaminase. His purest preparation catalyzed the consumption of one atom of oxygen per molecule of histamine inactivated. It would appear that, with purer preparations of histaminase for each molecule of histamine destroyed, there is a usage of one atom of oxygen and the formation of one molecule of ammonia and one molecule of aldehyde. After prolonged incubation with cruder kidney extracts, the imidazole ring is ruptured, and all 3 nitrogen atoms of the histamine molecule can be set free as ammonia. On

present evidence, it seems that the free amino group of the side chain is first removed, although Swedin,<sup>16</sup> working with a highly purified preparation (on present standards), considers the imidazole ring to be broken as a first step in the activation of histamine.

A variety of units of histaminase activity have been proposed. The original, proposed by Best and McHenry, was "that amount of activity which would destroy 1 mg. of histamine in 24 hours at 37°C. in a phosphate buffer at pH 7." A recent new unit is that proposed by Laskowski and his associates,<sup>17</sup> who claim it to be an advance over the older units because of the ease with which the unitage can be calculated from either the oxygen consumption or the liberation of ammonia. By definition, this new (Laskowski) unit is about 5.4 times larger than that of Zeller; 10 times larger than that of Stephenson;<sup>18</sup> 14 times larger than that of Best and McHenry; and 24 times larger than the "Torantil" unit.

Zeller considers histaminase to be a flavoprotein. He identified flavin after splitting the enzyme with acetone. Laskowski *et al.* were unable to confirm these findings. However, Kapeller-Adler<sup>19</sup> has prepared, from pig-kidney cortex, a powder possessing 1.5 to 2 units per mg. protein (Laskowski units). After dialysis during 1 to 3 days against tap water or phosphate buffer at pH 7.2, the activity of the preparation decreased towards histamine but increased towards cadaverine, putrescine, and agmatine as substrates. There was a loss of color of the enzyme preparation during dialysis, as in Zeller's experiments. When an acetone extract, yellow in color, of the original enzyme preparation was added to the dialyzed enzyme, the effects of dialysis were completely reversed. Kapeller-Adler has also been able to show that the prosthetic group of the flavoprotein, d-amino acid oxidase, namely, flavin adenine dinucleotide (F.A.D.), when added to dialyzed histaminase, was able to replace the yellow extract prepared from histaminase and equally well reversed the effects of dialysis. Further, the yellow extract from histaminase, added to the protein moiety of d-amino acid oxidase, was able to restore its activity. It appears, then, that the prosthetic group of histaminase is F.A.D. Kapeller-Adler suggests that F.A.D. may act as a coenzyme for the histamine-histaminase reaction but as an inactivator in the case of substrates such as cadaverine, putrescine, and agmatine, for F.A.D. (and the yellow extract) acts on undialyzed enzyme preparations, increasing the effect on histamine and decreasing the effect towards the other diamines used as substrates.

These experiments, quite apart from their innate interest, bring additional information with which to debate the nomenclature of this enzyme system. This work would seem to lend support to the retention of the term "histaminase" for these mammalian preparations rather than the more general term "diamine oxidase."

Marcou *et al.*<sup>20</sup> noted a considerable increase in the histamine-destroying power of the blood of pregnant women. This observation has been considerably extended, especially by Kapeller-Adler<sup>21</sup> and by Ahlmark,<sup>22</sup> who has also investigated similar changes in pregnant animals.

Ahlmark carried out his tests by a method in which the inactivation of

histamine incubated with the plasma is measured biologically. Kapeller-Adler has used a biological procedure, but, in much of her work, she has used a modification of the test of Zeller *et al.*,<sup>28</sup> in which the extent of the decolorization of indigo disulphonate, presumably due to the intermediary formation of hydrogen peroxide, is used as the measure of enzyme activity, while employing histamine as substrate. It would appear that the accuracy and general reliability of this chemical method is inferior to that of a carefully controlled biological assay.

According to Ahlmark, the histaminolytic power (which he considers to be due to histaminase) begins to increase in the serum of the pregnant woman about the 7th week after the last menstruation and reaches a peak at the 7th month. It falls rapidly after parturition. It is suggested that the increased histaminolytic power of the plasma in pregnancy is due to the passage of the enzyme from the placenta.

Danforth<sup>24</sup> reported the presence of histaminase in the human placenta and later put forward the tentative suggestion that the amount of histaminase in the placenta showed some correlation with the efficiency of uterine contractions. Kapeller-Adler,<sup>21</sup> in her investigations of 45 placentae, again pointed to a possible correlation between the amount of the histaminase in the placenta having been found to be inversely proportional to the uterine efficiency.

Ahlmark found values of the histaminolytic power of sera deviating from normal in some cases of pregnancy toxemia. There appeared to be no correlation between the degree of symptoms and the histaminolytic power. Kapeller-Adler's results are not in agreement with those of Ahlmark. She favors the assumption that histamine might be formed, in the metabolism of the pregnant woman, from the histidine, present in large amounts throughout gestation, by the enzyme histidine decarboxylase. In normal pregnancy, most of this histamine is presumably destroyed by the histaminase. In mild cases of pre-eclamptic toxemia, the activity of the histaminase may be impaired and the histamine may cause damage. In severe cases of pre-eclamptic toxemia and in eclampsia, the histaminase activity is very low, and the histamine formed in considerable quantities will then exert its toxic effect, more particularly on the kidneys. The role of diamine oxidase, or histaminase, in the tissues is still uncertain; this present evidence is very suggestive.

The report of Karady and Browne,<sup>25</sup> that the intravenous injection of histaminase (Torantil) could inhibit histamine shock and anaphylactic shock in guinea pigs, has not been substantiated by later workers using larger doses of the same or of more potent enzyme preparations.<sup>26-32</sup> It should be added, however, that Lemley and Laskowski<sup>33</sup> were able to protect one-third of their guinea pigs, which they had injected simultaneously with the enzyme and with one and one-half times the lethal dose of histamine. They were unable to use more enzyme preparation because of its toxicity, though, by present standards, its activity per mg. of protein was high.

It would be out of place in this paper to discuss even briefly the clinical results of the use of histaminase preparations. One significant observation

of Rose<sup>34</sup> may be mentioned: there was no effect on the histamine content of the blood of patients, following or during the administration of histaminase.

Stephenson,<sup>35</sup> in some preliminary studies, found that, when large doses of purified histaminase were injected intravenously into mice, it was possible to recover some of the enzyme in their tissues. A mouse receiving 65 units intravenously was killed 5 minutes later, and 90 per cent of the activity was recovered. When 130 units were given and the mouse killed one hour later, 50 per cent of the activity was recovered. The tissues of the mouse examined were blood, liver, and kidney. The enzyme was found in approximately equal amounts in liver and blood. None was recovered from the kidney. Stephenson presumed that the liver destroyed the injected enzyme.

Dale<sup>36</sup> first showed that adrenalectomy greatly enhanced the sensitivity of animals to injections of histamine. Many later workers have focused their attention on the part played by the adrenal cortex in controlling the effects of histamine, though it has been shown that the medulla, with its secretion of adrenaline, is also involved. The hypophysectomized animal is also more sensitive to histamine than is the normal animal, and, presumably, this difference may be ascribed to the accompanying atrophy of the adrenals. MacKay and Clark<sup>37</sup> found that the adrenal glands of rats given daily subcutaneous doses of histamine weighed 30 per cent more than those of controls and that the hypertrophy was confined to the cortex. A number of other experimenters, while producing a tolerance to histamine by repeated daily injections, have not been able to observe any increase in weight of the adrenal glands. It might be interjected here that the adrenals play only a minor role in the development of both tachyphylaxis and a tolerance towards histamine. A change in surface membrane conditions, as suggested by the experiments of Schild,<sup>38</sup> may well provide an explanation for these states (see also Dale<sup>39</sup>).

It has been shown by a number of independent workers that the resistance to histamine of the adrenalectomized animal may be restored to normal by the administration of cortical extract, though, as Rose<sup>40</sup> showed, considerably more than the maintenance dose of cortin for the adrenalectomized rat is required to restore the full ability of the animal to inactivate histamine. Karady *et al.*<sup>41</sup> observed a decrease in the histaminase content of the lung of adrenalectomized rats and that the enzyme could be restored to normal with adequate amounts of cortin. The histaminase content is considerably decreased if normal saline is not given in the diet of the adrenalectomized rats. When they are given normal saline, however, the enzyme content is only slightly decreased, and yet the rats are markedly sensitive to histamine when compared with the normal. It does not seem, as these workers pointed out, that the decreased resistance is due to the decrease in the histaminase, nor is it linked to a specific function of the adrenal glands. It is only one example of the generally decreased resistance of the organism under these conditions (see also Swingle and Remington<sup>42</sup>).

When anaphylactic shock was superimposed on histamine shock (in the

rabbit<sup>43</sup>), the removal of histamine was faster than when histamine alone was given, suggesting that some mechanism for the rapid destruction of histamine must be activated by the antigen-antibody reaction.

Rose and Browne<sup>44</sup> carried out an investigation on the rate of disappearance of intravenously injected histamine in the rat. In some experiments, 24  $\gamma$ /g. body weight were given and a succession of animals was killed at varying intervals of time after the injection. Within 15 minutes, relatively little histamine remained in the blood, the concentration of histamine being far higher in other tissues examined, namely, kidney, liver, lung, and lymph glands. The histamine content of the kidneys was particularly high, being 1600 times its normal value. In the interval between 15 and 30 minutes after injection, there was a sharp decrease in the histamine content of the liver. In the kidney, the decrease was only slight. Within 3 hours, all values approached the normal except those of blood and kidney. These authors suggested two possible explanations for the difference in the rate of disappearance of histamine from the various tissues studied. Assuming the presence of some mechanism in the kidney, other than histaminase, which is not present in the kidney of the rat, the fall of the concentration in the lung, liver, *etc.*, during the 15 to 30 minute period, and the absence of any decrease in the kidney are due to the continued transfer of the histamine from the other tissues to the kidney at such a rate that the destruction of histamine by the kidney is not apparent. If, however, one assumes that the main mechanism of histamine destruction is the histaminase action of those tissues which contain it, namely, lung and small intestine, the kidney in this case could be regarded as an organ which takes up a large amount of histamine, thus temporarily removing it from the circulation and slowly liberating it to be transferred to the histaminase-containing organs for destruction.

In summary, at present it would appear that the chief mechanism brought into play, as soon as there is an increase in the concentration of free histamine in the extracellular fluids of the body, is the direct attachment of histamine to cells, presumably to protein molecules of the cells which thereby anchor the histamine and temporarily render it inactive. Later, one presumes that the histamine is either inactivated, in the tissue to which it has become attached, or it is reversibly attached and, as the concentration of free histamine decreases in the surrounding fluid, it is carried to other inactivating tissues, which may derive at least some of their power of inactivation from their content of histaminase. It may be that histamine is also inactivated by mechanisms as yet unidentified.

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### Discussion

C. A. DRAGSTEDT (*Northwestern University Medical School, Chicago, Illinois*): There is a point in connection with the inactivation of histamine, which Dr. Waters has presented so ably, that merits mention because of its historical interest. A number of investigators, including Richard Weil in particular, made serious efforts to detect an anaphylatoxin (such as histamine) in canine anaphylactic shock by transfusing the blood of anaphylactic animals into normal animals. In view of the fact that anaphylactic reactions in the dog do not occur with an assured and predictable severity, they felt it necessary to wait about thirty minutes after the assaulting injection of antigen before withdrawing blood, in order that the effects of the blood-letting alone would not becloud or confuse an estimation of the severity of the anaphylactic reaction.

As we have since learned, this lapse of time was adequate for the complete disappearance of free histamine from the circulating plasma. As it is only the free histamine in the plasma which exercises physiologic effects, the negative results of such experiments are readily understood. In contrast, the relative ease with which we were able to demonstrate the liberation of histamine during anaphylaxis in the dog was due to the method which we first employed. Lymph was collected continuously from the thoracic duct, and, as histamine is inactivated at a negligible rate by lymph *in vitro* (or by dog's blood plasma *in vitro*, for that matter), our very first experiments were crowned with success. A similar explanation obtains for the positive results which Bartosch, Feldberg, and Nagel achieved when they perfused the lungs of anaphylactic guinea pigs and detected a histamine-like substance in the perfusate. It was only after there was due appreciation of the astonishing speed with which free histamine disappears from the circulating blood that it was possible to demonstrate substantial amounts of histamine in the blood of these animals during anaphylaxis.

# HISTAMINE-PROTEIN COMPLEXES IN ANAPHYLAXIS AND ALLERGY. A REVIEW

By Norbert Fell\*

*Camp Detrick, Frederick, Maryland*

The synthesis of histamine-azoprotein was attempted several years ago in an effort to discover if a histamine-protein complex could be used to create an artificial immunity to histamine in animals and man. If histamine-specific antibodies could be developed, it was postulated that they might bind any histamine released during an antigen-antibody reaction *in vivo*. The bound histamine might not be able to reach or affect the shock organs, and thus some of the typical symptoms of anaphylaxis and allergy might be prevented. This hypothesis was based, of course, on the original important observations of Dale and Laidlaw,<sup>1</sup> as well as those of Lewis,<sup>2</sup> and the fundamental work of Landsteiner in the field of immunologic specificity.

The method of synthesis and the immunologic properties of several histamine-azoproteins<sup>3, 4</sup> (differing only in the protein components) and of a histamine-isocyano protein<sup>5</sup> have been published. This work was interrupted by the war and has been resumed only in a limited form. Some of the published material and certain earlier work which did not reach a stage justifying publication will be described in this review.

The synthesis of the first histamine-azoprotein presented some difficulties, but these were finally overcome and the compound was obtained in the desired form. The details of the synthesis have been described previously.<sup>4</sup> The protein used most frequently was despeciated normal horse-serum globulin prepared by the Taka-diasase antitoxin concentration process of Coghill and Fell.<sup>6</sup> This protein was employed so as to eliminate, as far as possible in clinical studies, any risk of sensitization to intact horse-serum protein, in case the whole theory of effective immunization by histamine-azoprotein proved to be unsound. Despeciated normal horse-serum globulin has had most of its horse-serum specificity removed by the treatment with Taka-diasase.

In laboratory experiments, other proteins frequently were used for coupling. These included casein, normal horse-serum globulin, cow-serum globulin, and dog serum. The histamine-azoprotein solution was diluted to contain about 2 per cent of the complex, the pH adjusted to 7.6, phenol added to a concentration of 0.5 per cent, and the solution sterilized by Mandler filtration. The amount of bound histamine in such a solution was usually about 1 mg. per ml. The histamine in the final product was firmly bound and no tests for free histamine were ever positive. The bound histamine could be liberated, however, by hydrolysis of the product with concentrated hydrochloric acid. After such hydrolysis the liberated histamine could be assayed by a method such as that of Code.<sup>7</sup>

\* The investigations described in this paper were carried out in the Research and Biological Laboratories of Parke, Davis & Company, Detroit, Michigan.



The immunologic specificity of histamine-azoprotein was tested mainly with antisera from rabbits hyperimmunized with histamine-azo-despeciated-horse-serum globulin. Antisera with fairly high precipitin titers could be obtained only by immunizing rabbits intensively by intravenous injection for a period of 6 to 8 weeks. The observed weak antigenicity of the complex is in agreement with observations by Landsteiner and VanderScheer,<sup>8</sup> Hooker and Boyd,<sup>9</sup> and others that basic haptens such as histamine are

TABLE 1  
PRECIPITATIVE TESTS WITH RABBIT ANTISERUM TO HISTAMINE-AZO-DESPECIATED-HORSE-SERUM GLOBULIN

Rabbit no.	Antigen dilutions							Normal rabbit serum 1-50
	1-10	1-50	1-100	1-200	1-400	1-800	1-1600	
1	++++	+++	+++	++	+	+	±	—
2	++++	+++	++	+	+	+	±	—
3	+++	++	++	++	+	+	±	—
7	++++	+++	++	++	+	+	—	—
8	++++	+++	++	++	++	+	+	—
10	+++	+++	++	++	+	+	±	—

0.2 ml. of the antigen dilution and 0.2 ml. of the antiserum mixed, left for 1 hour at 37°C., 18 hours in the refrigerator, and read.

TABLE 2  
CROSS PRECIPITATIVE REACTIONS OF ANTIHISTAMINE-AZO-DESPECIATED-HORSE-SERUM-GLOBULIN-RABBIT-SERUM WITH VARIOUS HISTAMINE ANTIGENS

Dilution of Antigen	Antigens Tested			
	Histamine-azo-despeciated-horse-serum globulin	Histamine-azo-rabbit serum	Histamine-azo-casein	Despeciated horse-serum globulin
1-80	+++	++	±±	++
1-160	++	±±	+	+
1-320	±±	+	—	+
1-640	+	—	—	—
1-1280	—	—	—	—

Controls = antigen + saline  
antigen + normal rabbit serum  
antigen + normal horse serum  
immune serum + saline

} negative

All antigens prior to dilution contained 5-6 mg. N per ml.

inferior to acidic in the production of specific antisera. Results of a typical precipitative test with rabbit antiserum are shown in TABLE 1 and, as can be seen, definitely positive tests usually were obtained only at antigen dilutions not higher than 1:800. However, most of these rabbit antisera produced cross reactions with histamine-azoproteins made with heterologous protein components. Results of a typical test of this type are given in TABLE 2. Precipitation with the homologous protein was also obtained, indicating that the specificity of this protein had not been completely altered

by combining it with para-aminobenzoyl histamine. The results of the inhibitive test shown in TABLE 3 give another indication of the partial specificity of antibodies produced by histamine-azoproteins. These results show inhibition of precipitin reaction by histamine and some inhibition by para-aminobenzoyl histamine and histamine azocasein. Compounds closely related chemically, such as tyramine and histidine, however, did not have any inhibitive capacity.

A study of protection against anaphylaxis by prior immunization with the histamine-protein complex was carried out in guinea pigs and rabbits. The procedure consisted of immunization of a series of animals with the complex, followed by sensitization to crystalline ovalbumin. A series of controls was also sensitized, following which all the animals were injected with shocking doses of ovalbumin.

Some results of the direct anaphylaxis experiment with guinea pigs are shown in TABLE 4. Immunization consisted of three subcutaneous injections

TABLE 3  
INHIBITIVE TESTS WITH RABBIT ANTIHISTAMINE-ANTIGEN SERUM

Time	Precipitative reaction after incubation with inhibitors											
	Saline		Histamine HCl		P-NH <sub>2</sub> benzoyl histamine		Tyramine HCl		Histidine HCl		Histamine-azo-casein	
			20 mg.	10 mg.	20 mg.	10 mg.	20 mg.	10 mg.	20 mg.	10 mg.	10 mg.	5 mg.
minutes												
30	3+	3+	—	2+	—	3+	3+	3+	+	+	—	+
50	4+	4+	—	3+	+	4+	3+	4+	2+	3+		
70	4+	4+	—	4+	3+	4+	4+	4+	3+	4+		
120	4+	4+	—	4+	4+	4+	4+	4+	4+	4+		

0.2 ml. antiserum 1:28 incubated with 0.2 ml. inhibitors at 37 C. for 1 hour and 0.4 ml. of histamine-azohorse-serum-globulin then added.

weekly of histamine-azoprotein: 0.5 ml. the first week, 1.0 ml. the second, and 1.5 ml. the third week. The control animals were treated similarly with equivalent amounts of despeciated horse-serum globulin.

Both series were then sensitized with three doses of 5 mg. of crystalline ovalbumin given subcutaneously at two-day intervals. One day after the last albumin injection, the treated series received an injection of 1 ml. of histamine azoprotein and the controls were given an equivalent amount of despeciated, normal horse-serum. One week later, this immunizing dose was repeated. After four weeks, all the animals were given shocking doses of albumin. It is evident that some definite protection against anaphylactic shock is produced by this treatment. Statistical analysis showed that the differences in anaphylactic reaction between the two groups were significant.

Anaphylaxis in rabbits immunized with histamine-azoprotein and sensitized to ovalbumin was investigated by checking blood pressure before and during the period of shock produced by intravenous injection of ovalbumin.

The blood pressure effects were not particularly significant, although, in the control group, 5 of 8 rabbits had a pronounced drop below normal and there were 3 deaths. Four of 6 immunized animals showed only a slight rise in pressure with slow return to normal; the other two exhibited little or no change in blood pressure.

TABLE 4  
ANAPHYLAXIS IN GUINEA PIGS IMMUNIZED WITH HISTAMINE-AZOPROTEIN AND  
SUBSEQUENTLY SENSITIZED TO OVALBUMIN

Control animals		Histamine-azoprotein-treated animals	
Crystalline ovalbumin, 10%	Anaphylaxis	Crystalline ovalbumin, 10%	Anaphylaxis
<i>ml.</i>		<i>ml.</i>	
A 1.0 I.A.	++	2.0 I.A.	±
1.5 "	+++	2.0 "	±
2.0 "	++++	3.0 "	±
2.0 "	+++	3.0 "	±
3.0 "	++++	3.0 "	++
3.0 "	++++	3.0 "	+
3.0 "	++++	3.0 "	+
3.0 "	++++	3.0 "	+
B 5.0 "	++	5.0 "	++
2.0 "	++	0.5 I.V.	+
2.0 I.V.	++++	1.0 "	++
1.0 "	++++	3.0 "	++
C 1.0 I.A.	++	3.0 I.A.	±
2.0 "	++++	3.0 "	±
2.0 "	++	3.0 "	±
3.0 "	++++		
3.0 "	++++		
3.0 "	++++		
2.0 "	++		
50% fresh albumin		50% fresh albumin	
D 5.0 I.A.	++++	4.0 I.A.	+++
3.0 "	++	4.0 "	++++
4.0 "	++++	4.0 "	++++
4.0 "	++++	4.0 "	+++
4.0 "	++	4.0 "	++++
4.0 "	++	4.0 "	+++
5.0 "	±	4.0 "	+
4.0 "	+++	4.0 "	+
		4.0 "	+

++++ = fatal anaphylaxis with typical symptoms and pathological findings on autopsy.  
I.A. = intra-abdominally; I.V. = intravenously.

Blood was taken from the hearts of immunized and sensitized rabbits prior to the administration of the shocking dose of ovalbumin, and this blood was used for experiments on *in vitro* liberation of histamine according to the method of Katz.<sup>10</sup> A summary of all experiments of this type showed that the plasma of 13 of 17 control animals yielded one microgram per ml. or more of released histamine, while in the plasma of only 3 of 17 immunized animals was there a similar increase in histamine. These differences were also shown to be significant by statistical analysis. The trypan-blue test

that has been employed by Ramsdell<sup>11</sup> and Rocha e Silva and Dragstedt<sup>12</sup> for the identification of histamine liberation in tissues was also employed with rabbits immunized to histamine-azoprotein and subsequently sensitized to ovalbumin. In a small series of animals, normal controls sensitized to ovalbumin gave good positive trypan-blue results when tested with ovalbumin and also with histamine, whereas the immunized rabbits showed negligible histamine release on injection of ovalbumin and a considerably smaller response to histamine than was the case with the controls.

The partial specificity for histamine of antibodies to histamine-azodespeciated-horse-serum globulin has been confirmed by Rosenman and Starin,<sup>13</sup> using laboratory animals, and also to some extent by Cohen,<sup>14-17</sup> with human sera from patients treated with the histamine-protein complex. Using the iontophoresis technique with mixtures of histamine and serum from treated patients, he frequently could demonstrate that considerably lower dilutions of histamine were required to produce whealing than was the case with histamine in saline or normal serum. Where the threshold to histamine was normally 1:640,000, dilutions of the compound in treated serum necessary to produce wheals varied from 1:64,000 to the normal dilution. Two of these antisera were sent to Katz,<sup>18</sup> who reported that one of them neutralized 25 per cent of added histamine base when tested by his method on the guinea-pig ileum. Cohen, using 7 human sera and testing for precipitins by the Goodner method, found that all but one were 2 to 4 plus at 1 to 160, and 4 of the 7 were 3 or 4 plus at 1 to 640. One of these sera also gave 3 plus precipitation at a dilution of 1 to 128 with histamine-azocasein. With some of these human sera the inhibition reaction could be demonstrated.

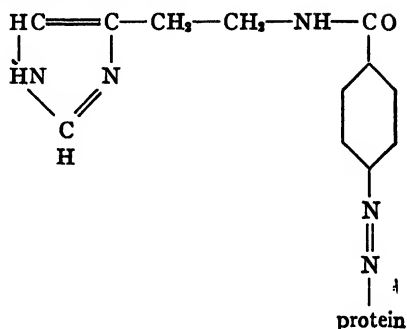
However, in all the clinical and laboratory reports that have accumulated in the literature, the relatively poor antigenicity of histamine-azoprotein has been evident. Large dosages at frequent intervals for a comparatively long period of time have been necessary in order to produce demonstrable antibodies in animals and man.

One of the interesting aspects of immunization with histamine-azoprotein is that the antibodies produced do not seem to interfere in any way with the normal functions of histamine in animals or man. Laboratory and clinical studies have indicated that immunization with this compound has no effect on gastric secretion, capillary dilatation, or blood pressure. Similar results were obtained by Clutton<sup>19</sup> with thyroxin globulins and by Went<sup>20</sup> with adrenalin azoprotein. None of the results reported by these workers indicated any effect on the normal function in the animal body of the chemical agent that was acting as a hapten in the complex antigen.

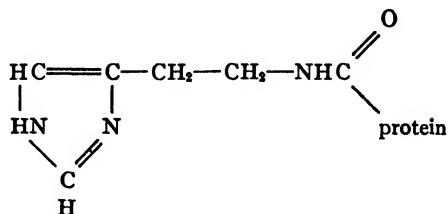
Other types of histamine-protein complexes have been prepared, and details of the synthetic methods described.<sup>21</sup> None of these complexes other than H-isocyanoprotein was tested immunologically, but it might be interesting for someone to investigate them, particularly those where protein was coupled with histamine at some point other than through the free amino group. A list of these complexes, including histamine-azoprotein, is given in TABLE 5.

TABLE 5  
HISTAMINE-PROTEIN COMPLEXES

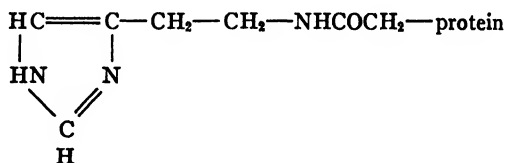
Histamine-azo-despecciated-horse-serum-globulin



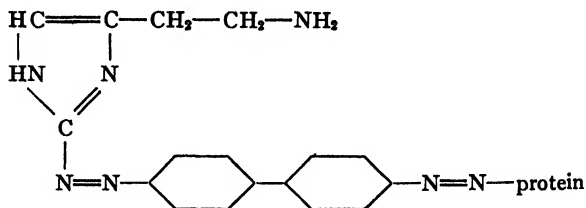
Histamine-isocyano-protein



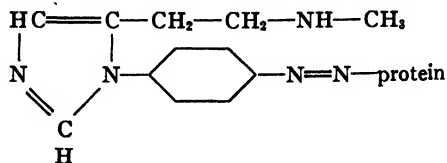
Complex from chloracetyl histamine



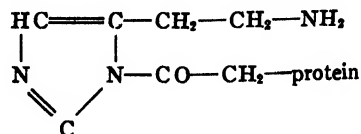
Complex from 2-(azobenzidine azo proteo-) histamine



Complex from 5-(β-methylaminoethyl)-l-imidazyl-p-benzene diazonium chloride



Complex from 5-(β-carbobenzoxy-amidoethyl)-l-chloracetyl imidazole



The clinical reports<sup>3, 14, 16, 17, 22-32</sup> on histamine-azoprotein indicate a marked discrepancy in the results obtained. A number of physicians have reported using the compound with excellent results in cases of atopic eczema, contact dermatitis, urticaria, physical allergy, allergic rhinitis, histamine headache, allergy to flea bites, and sensitivity to liver extract. On the other hand, some clinicians have reported little or no benefit from the use of the compound. Furthermore, some fairly severe local and a few systemic reactions have been reported during recent years following the injection of histamine-azoprotein. This is somewhat surprising, because, during all the early clinical work with the compound, practically no reactions were seen other than mild local reactions and an intense area of local redness when the complex was injected intracutaneously. In no case was there a reaction which might be ascribed to sensitization to histamine-azoprotein itself. There were 2 or 3 instances where patients experienced some degree of what appeared to be histamine shock almost immediately after injection of the complex. It was felt that certain individuals might have a peculiar enzyme system that could liberate histamine from the complex. It seems possible that some of the later reactions reported may have been of this nature.

During recent years, attempts have been made to increase the antigenicity of histamine-azoprotein. These involved combinations of the complex with adjuvants and also adsorption of histamine-azoprotein on alum, calcium phosphate, or aluminum phosphate. Immunization with these materials usually shortened the time required for precipitins to appear in rabbit serum, but in no case was the precipitin titer raised appreciably over that which could be obtained with histamine-azoprotein itself. Another method tested involved the addition of more tyrosine by coupling on the acid azide to despeciated normal horse-serum globulin. This compound did not give evidence of any increase in antigenicity.

It was found recently that despeciated normal horse-serum globulin had considerably more tyrosine than did the untreated protein. The comparative amounts were 21 and 6.7 per cent. On this basis, therefore, it theoretically should be possible to attach more histamine to the protein molecule than had been assumed initially, and this proved to be true. However, the histamine complex, with considerably greater histamine content than the usual compound, was not improved in antigenicity.

Coffin and Kabat<sup>33</sup> have conducted an interesting investigation on histamine-azoprotein, using passively sensitized guinea pigs in protection experiments. They found that guinea pigs immunized with the complex showed little or no protection against 1 MLD. of histamine injected intravenously. We had made a similar observation, but we did notice some slight protection in immunized rabbits, as judged by the decreased fall in blood pressure, compared with that of control animals following the intravenous injection of histamine. We assumed that the lack of protection against histamine could be explained, as Coffin and Kabat have suggested, on the basis that histamine released during anaphylaxis is developed more slowly, at different sites, and in different quantities from that injected intravenously.

It seems probable that intravenous injection permits histamine to reach the organs of shock before there is time for antibodies to neutralize it.

Coffin and Kabat also found that guinea pigs immunized with histamine-azoprotein were protected against subsequent anaphylaxis, but that control animals immunized with human serum were similarly protected. This is an interesting finding in view of the fact that our control animals in anaphylaxis experiments with guinea pigs were always immunized with despeciated normal horse-serum globulin equivalent to that contained in the complex given the treated guinea pigs. Our control group received practically no protection from the protein itself. Of course, we were dealing with active sensitization, whereas the experiments of Coffin and Kabat involved the passive method. It would be interesting if their experiments could be repeated using actively sensitized guinea pigs.

Coffin and Kabat have also reported, on the basis of 4 guinea pigs, that active sensitization to histamine-azoprotein was possible. No control animals were mentioned. In our experience, we never could produce anaphylaxis in guinea pigs actively sensitized to the complex until we reached a dosage that intravenously would kill normal guinea pigs. We assumed, therefore, that deaths occurring in both sensitized and normal animals were really anaphylactoid in nature.

A survey of all the literature that has accumulated about histamine-azoprotein leads to the conclusion that the compound has helped support the theory that histamine plays some role in anaphylaxis and allergy. The results obtained with the antihistamine drugs, such as Benadryl, Antergan, and Pyribenzamine, have almost forced acceptance of the histamine theory, at least to the extent that histamine almost certainly must be considered one of the factors involved in anaphylactic and allergic reactions. A summary of all the clinical reports that are available about the antihistamine drugs and histamine-azoprotein indicates that the percentage of beneficial clinical results is approximately the same for all these compounds. Moreover, all of these antihistamine agents seem to be most effective clinically in the same types of allergies, namely, urticaria, atopic dermatitis and eczema, vasomotor rhinitis, and physical allergies, while none seems particularly beneficial in bronchial asthma. It seems possible that, in certain types of allergy, histamine may play a more dominant role than in others, but it is also likely that, in practically all forms of allergic reactions, there are other factors involved in addition to histamine.

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*Discussion of the Paper*

R. A. COOKE (*Roosevelt Hospital, New York, N.Y.*): My remarks will be directed primarily toward the use of histamine azoprotein by physicians in clinical allergy rather than to immunochemical aspects.

The theory on which Dr. Fell<sup>1, 2</sup> and his associates proceeded in the preparation of histamine azoprotein was an interesting one, for, while there is still no absolute proof, evidence is accumulating that histamine probably is liberated in anaphylactic reactions as one of the active substances responsible for the symptoms. Purely by analogy, therefore, it may be important also in certain of the clinical allergies. To this point I shall return later, for all allergies are not identical.

Dr. Fell states that the clinical reports indicate a marked discrepancy in results obtained. Actually, the number of authoritative clinical reports are few, although it is known that the commercial preparation, Hapamine, was extensively used by physicians generally. After several years of study, Sheldon *et al.*,<sup>3</sup> reporting on thirty-nine cases, were able to say no more than that the results were "sufficiently encouraging to justify a continuation of the study." In 1943, Cohen and Friedman<sup>4</sup> presented serologic studies of a few patients treated with histamine azoprotein, but did not pretend to furnish evidence of any therapeutic effect. Their last contribution<sup>5</sup> on histamine azoprotein therapy, however, was limited to fifty-five cases, with 50 per cent negative results. Dundy, Zohler, and Chobot<sup>6</sup> concluded that "treatment with histamine azoprotein was generally ineffective in a series of forty allergic individuals." A small therapeutic trial with serologic studies, conducted at my clinic at Roosevelt Hospital with Hebdal and Downing,<sup>7</sup> led us to conclude that histamine azoprotein did not protect patients with pollen hay fever, did not prevent the induced constitutional reactions (analogue of anaphylactic shock) in ragweed-sensitive patients, and did not alter the level of the cutaneous reaction on intradermal test to the histamine base. Although precipitins to the histamine azoprotein were found in the blood of five of eight patients, in no case could we demonstrate a precipitate to casein-azoprotein. This I believe to be the extent of published clinical studies. They are very meager and, as far as they go, do not support a claim for very beneficial therapeutic effects. Further, the rapidity with which this compound has disappeared from the therapeutic arena would indicate that it proved useless for treatment of allergies in the hands of the medical profession generally.

There is another aspect, however, which I feel warrants mention. Granted that histamine may be a factor in allergies of the immediate wheal type, the one most likely based upon a mechanism similar to that of anaphylaxis (*e.g.*, hay fever, urticaria, and certain of the asthma group), one must, I believe, differentiate sharply between these and those allergies of the so-called "delayed group," in which belong dermatitis of external or internal origin, certain vascular reactions, and the tuberculin type of bacterial allergy. The reasons are: (1) the histologic response in these latter reactions bears no relation to the pharmacologic effect which histamine is known to produce in tissues; and (2) the antibody mechanism is different.

Bearing this point in mind, if we again review the recorded studies, it appears that a large number of the treated patients exhibited allergies of the delayed type. It was in them that most of the best results were obtained by Sheldon *et al.* and by Cohen and Friedman. As a matter of fact, the latter authors state "it (histamine azoprotein) is of no value when used alone in the prevention of hay fever" (which is typical of the immediate allergies that might be produced by liberation of histamine). The interpretation of therapeutic results is hazardous business, and the results just referred to and attributed to the drug used (histamine azoprotein) merely indicate to my mind the difficulties and fallacies inherent in clinical studies generally.

It needs only to be mentioned that histamine azoprotein is antigenically active, as proven by Coffin and Kabat,<sup>8</sup> and not devoid of danger in the treatment of patients.

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S. B. HOOKER (*Boston, Massachusetts*): One aspect of this topic that has not been given adequate consideration is the neutralizing strength of anti-haptenic sera which can be produced by immunization with proteins coupled with pharmacologically active substances of low molecular weight. About fifteen years ago, Dr. Boyd and I attempted unsuccessfully to produce antimorphine. Later, we did obtain several antisera from rabbits prolongedly immunized with a strychnine-hemocyanin-diazonium compound. These sera were weak but specifically precipitated with casein-azo-strychnine. This aggregative property was specifically inhibited by various salts of strychnine and by brucine, but the sera failed entirely to protect mice against a single lethal dose of strychnine.

During that period, we considered the possibility of producing a specific antihistaminic serum for the purpose of combating shock, but a simple calculation dissuaded us from making the attempt. These azoproteins are poor antigens. With us and with others, the amount of antihapten they had produced was measured in micrograms rather than milligrams. When

we made the liberal assumption that sera ten times stronger than ours might be obtained and, further, over-liberally allowed *quadri*-valence to each molecule of antihapten, as well as irreversible combination, computation revealed that about three liters of immune serum would be required to neutralize a single average dose of morphine lethal to man. It is well known, of course, that a relatively enormous number of simple haptenic molecules is required to inhibit antihaptenic sera, and this indicates a high dissociation constant, probably increasing as the molecular size (and complexity) of the hapten diminishes. Histamine is about one third as large as morphine.

For these and other reasons, the production of an antihistaminic serum did not promise much in the way of practical usefulness, which, however, does not derogate from its theoretical interest.

# INTRODUCTION TO ANTIHISTAMINE AGENTS AND ANTERGAN DERIVATIVES

By Daniel Bovet

*Laboratorio di Chimica Terapeutica, Istituto Superiore di Sanità, Rome, Italy*

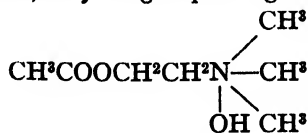
The scope of therapeutic chemistry can be characterized by the problem it intends to solve, which is essentially the study of the relationship between molecular structure and physiological activity. This field of chemical therapeutics may be represented by a large checkered table, with the pharmacodynamic properties of the agents under consideration being placed on the axis of ordinates and the long list of known chemical products on the axis of abscissas. It may be seen at once that, in either direction, developments are practically unlimited.

In the present state of our knowledge, it is possible, in certain fields of pharmacology, to evaluate the data so obtained and thereby establish certain methods of approach which may be applied in a general way. I intend to illustrate these various methods of approach, selecting as an example the history of the investigations on antihistamine substances, a subject which has demonstrated with particular sharpness how integrated have been the efforts of pharmacologists and chemists.

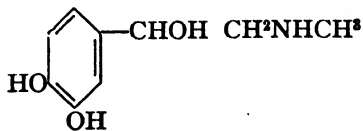
These are the points to be illustrated in this paper. A report will be made on the results of investigations carried out since 1937 by numerous research workers, in various countries, on antihistamine substances.

Antihistamine drugs<sup>1</sup> represent a group of synthetic amines displaying in most animals species, as well as in man, an antagonism specifically directed against the physiological and toxic effects of histamine. Perhaps no better definition could be given of an antihistamine substance than to state that it is a counterpoison having no specific activity of its own on the normal animal, its properties appearing only when it can manifest a detoxifying power against the action of histamine. The great activity of antihistamine agents is not due to any spasmolytic, sympathomimetic, or analeptic properties. In experimental pathology and human therapeutics, antihistamine substances are active against a large number of disturbing manifestations, particularly against anaphylactic reactions, which in many instances have been attributed to the release of histamine in the tissues.

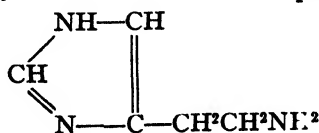
Three naturally occurring amines, acetylcholine, epinephrine, and histamine, may be grouped together—



acetylcholine



epinephrine



histamine

—because they have a similar chemical structure, are all present in the body fluids, and exert characteristically strong pharmacologic activities.

There are alkaloids which interfere with the effects of acetylcholine. Similarly, there are sympatholytic poisons which neutralize or reverse the effects of epinephrine. It seemed possible to me, therefore, that some substance might exist which exerts a specific antagonism toward histamine.

With this hypothesis in mind, I began an investigation in 1937 to determine the effect upon the susceptibility toward histamine of various substances of known activity on the autonomic nervous system. The results obtained with a series of phenolic ethers, including substances which possessed either antiepinephrine or antihistaminic activity, led to the first confirmation of the validity of this assumption. Since then, compounds

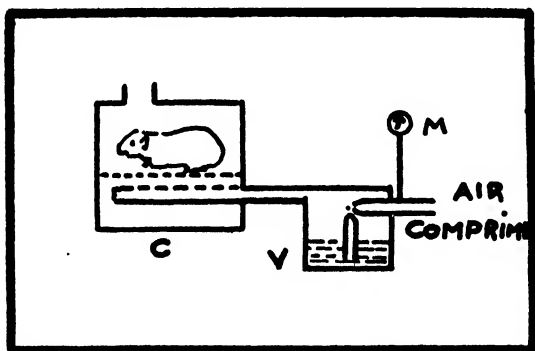


FIGURE 1. Diagram of an apparatus in which bronchospasm in guinea pigs is produced by inhalation of histamine aerosol. C—closed box in which the animal is observed; V—aerosolizer containing a solution of histamine hydrochloride; M—manometer (Bovet and Walthert<sup>21</sup>).

with similar or greater activities have been found in approximately ten different chemical series.

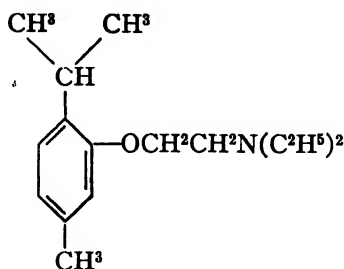
A certain number of laboratory methods have been proposed for the evaluation of the degree of activity of the various compounds. In the first test, we determined the action against the lethal effects of histamine in guinea pigs. This test is perfectly specific. In the second test, we determined the protection against histamine administered in the form of an aerosol. Here, symptoms similar to asthma are produced (FIGURE 1). A third method for determining antihistaminic activity, the least specific one, consists in ascertaining, *in vitro*, the effect on histamine-induced spasm of the isolated guinea-pig ileum.

First of all, it is important to note that many substances with a widely differing chemical constitution exhibit antihistamine properties, but that only few substances present this property to a high degree. Thymoxyethyl-diethylamine, or 929 F,\* is the first of the substances in which antihistamine properties were recognized.

From the chemical standpoint, Compound 929 F belongs to a series of amines with a phenolic ether function. It is generally known that it was

\* F = Fourneau.

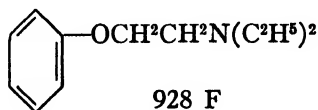
Fourneau (chief of the Department of Therapeutic Chemistry in the Pasteur Institute, and with whom I had the privilege of close association for twenty years), who, in 1910, was one of the first to synthesize the amines of this group.<sup>2</sup>



929 F

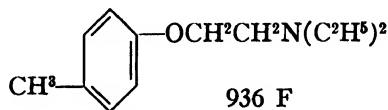
Eichholz,<sup>3</sup> in 1928, called attention to the properties of Gravitol, and Anan,<sup>4</sup> in 1929, described similar properties of Tastromine or thymoxyethyl-dimethylamine. Since 1933,<sup>5</sup> further derivatives were prepared in France, where particular attention was drawn to the sympatholytic activity of Prosympal (883 F<sup>6</sup>). Other derivatives of the same group are sympathomimetic or nicotinic or else they exert an activity which mainly influences the heart.<sup>7, 8</sup>

In 1937, a thorough investigation of 929 F, carried out with Staub, who was then preparing her doctorate thesis in my laboratory, made it possible to show: (1) the protection conferred to guinea pigs which had received several lethal doses of histamine;<sup>9</sup> (2) the antagonism directed against the histamine-induced spasms of smooth muscles of the intestine, stomach, uterus, and bronchi;<sup>9, 10</sup> and (3) the protection against experimental anaphylactic shock.<sup>11</sup> These first results have been confirmed and enlarged by several authors, more especially in the United States by Rosenthal and



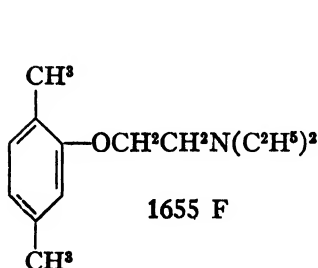
928 F

Protection: none



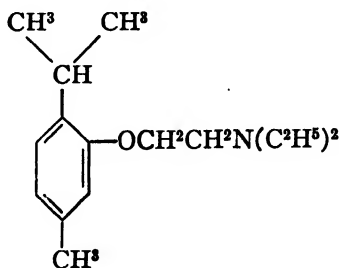
936 F

Protection: none



1655 F

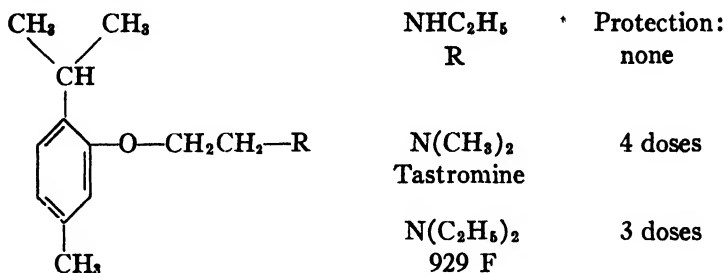
Protection: 2 doses



Protection: 3 doses

Minard,<sup>12</sup> Brown,<sup>13</sup> Lambert,<sup>14</sup> Climenko, Hamburger, and Messer,<sup>15</sup> Burchell and Varco,<sup>16</sup> Loew and Chickering,<sup>17</sup> Bourque,<sup>18</sup> and Hallenbeck.<sup>19</sup>

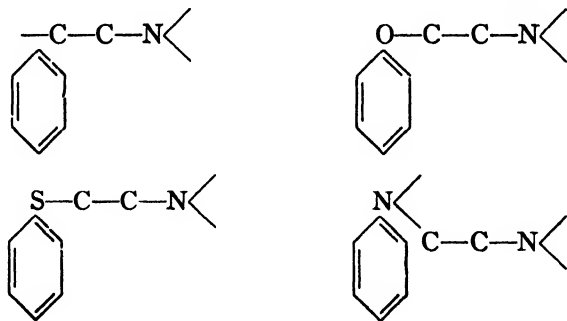
Thymoxyethyl-diethylamine (929 F) is the most interesting substance of antihistaminic activity within the phenolic ether series. Staub<sup>10</sup> has shown that antihistamine activity is strongly influenced by substitutions on the nucleus. Tastromine, a dimethylated homologue of 929 F., is probably



somewhat more active. On the other hand, the corresponding derivative with a secondary amino group is totally ineffective.

Through the experimental research on thymoxyethyl-diethylamine, it was possible to ascertain for the first time that there exist substances which exhibit antagonistic activity towards histamine.

Previous studies of sympathomimetic and sympatholytic poisons, carried out in the Pasteur Institute, had shown that a relationship exists in the pharmacodynamic responses between the different series of alkyl-aromatic amines: phenylethylamine, phenoxyethylamine, phenylthioethylamine, and phenylethylenediamine.<sup>8</sup> It seemed obvious, then, to apply our improved

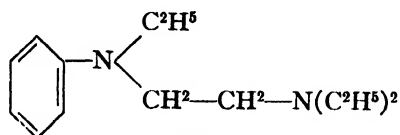


techniques of antihistamine assay to these series. Since neither phenylethylamines nor thioethylamines counteracted histamine, we turned to the study of aniline compounds. Derivatives of aminoethylaniline had been the object of limited study before we had found their antihistaminic activity.

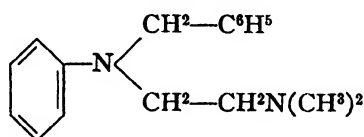
A compound of this type is described in a work by Weese<sup>20</sup> dealing with analeptic research on Icoral. Its cardiotonic action is mentioned in a paper by Pratesi.<sup>21</sup> Synthetic research undertaken in Fourneau's laboratory by de Lestrangé and J. P. Fourneau<sup>22</sup> has disclosed that, in this series, sympa-

thomimetic, sympatholytic, and antihistaminic amines are found side by side.

One derivative, diethylaminoethyl-N-ethyl-aniline, (designated 1571F), has proven to be more effective than 929F.<sup>10</sup> Our results published in 1939 have been confirmed in America by Burchell and Varco,<sup>16</sup> Loew,<sup>17, 23, 24</sup> Bourque,<sup>18</sup> Wilcox and Seegal,<sup>25</sup> *et al.* and in France by Halpern<sup>26</sup> in 1940, 1941, and 1942.



1571 F

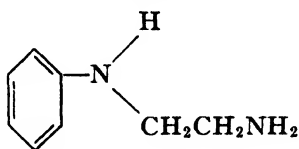


Antergan

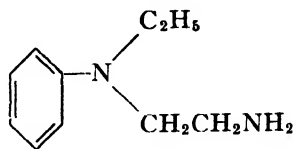
In 1942, a dimethylamine derivative corresponding to 1571F, N-diethyl-aminoethyl-N-benzyl-aniline (Antergan), was prepared by Mosnier and investigated by Halpern.<sup>26</sup> Utilizing the aforementioned toxic-dose test in guinea pigs, he showed that this derivative was approximately ten times more active than 1571F. Antergan, now replaced in France by Neo-antergan, was the first antihistamine agent commonly employed in therapy.

Considerable data are now available on the relationship between pharmacodynamic activity and chemical structure in the aniline series. To the number of substances investigated by Staub should be added those studied by Halpern<sup>26</sup> and a great number described more recently by Viaud<sup>27</sup> in the laboratories of the Société Rhône-Poulenc, who has thoroughly investigated the chemical aspects of this problem.

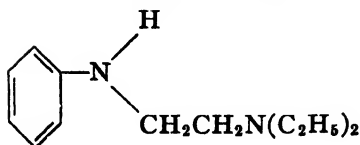
Starting with the basic structure common to both the molecule of 1571F and Antergan, it is a simple matter to follow the variations in activity brought about by molecular transformations.



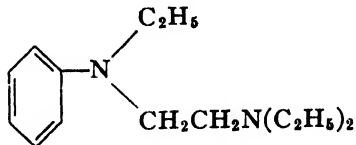
Protection: none



none



little



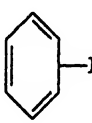
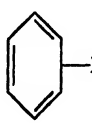
4 doses



The part played by substitutions on the amino groups, is demonstrated by the antihistamine effect of the four derivatives investigated in my laboratory, as shown in the previous diagram.

Antihistamine activity only appears when both amino groups are tertiary. Aminoethylaniline is inactive against histamine, although it is a hypertensor and a bronchodilator.

Besides the primary, secondary, or tertiary character of amines, the weight of the substituents introduced into the molecule predominantly influences the degree of antihistamine activity. It was shown that the substitution

		
	2325 RP	1571 F
Protection secured against histamine shock (guinea-pig)	10 doses	4 doses
Protection against the toxic effects of a histamine aerosol (guinea-pig) mg/kg	5 mg	20 mg
Spasmolytic activity on the isolated intestine (2325 RP = 1)	1	$\frac{1}{4}$

of dimethylamine for diethylamine in the side chain increased the activity of 1571 F. approximately two and one-half times.

Of no less importance is the effect of substitutions made on the aromatic amino group. The activity, quite insignificant when R is substituted by

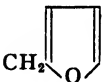
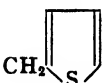
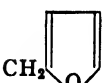
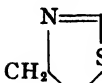
$\text{C}_6\text{H}_5\text{N}(\text{R})(\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2)$

Dose of antihistamine drug  
permitting to effect  
prevention of bronchospasm  
caused by inhalation of a  
histamine aerosol (mg./kg.)

Spasmolytic activity  
on the isolated  
intestine  
of guinea-pig  
(Antergan = 1)

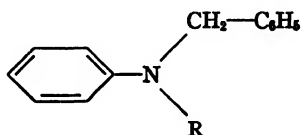
(a)

H	no action	1/100
CH <sub>3</sub>	no action	1/100
C <sub>2</sub> H <sub>5</sub> (2325 RP)	5	1/15
n C <sub>3</sub> H <sub>7</sub>	2.5	1/8
n C <sub>4</sub> H <sub>9</sub>	2.5	1/4
n C <sub>6</sub> H <sub>11</sub>	2.5	=
n C <sub>6</sub> H <sub>13</sub>	8	1/10
n C <sub>7</sub> H <sub>15</sub>	50	1/30

(b)	$C_6H_5$	50	=
	$CH_2 \cdot C_6H_5$ (Antergan)	1	1
	$CH_3CH_2 \cdot C_6H_5$	no action	
(c)		1	1
		no action	
		20	1/20
		1	1/1.6

a simple methyl group, becomes greater as the molecular weight of the substituents is increased to an optimum with five carbon atoms. The maximum activity is reached with the benzyl radical. In addition to the hydrocarbon chains, many other groupings and nuclei have been introduced.

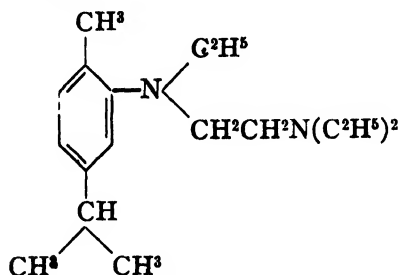
Moreover, the length of the aliphatic side chain appears to be a determining factor. Its role is similar to that which pharmacologists have observed



	Dose of antihistamine drug permitting to effect prevention of broncho-spasm caused by inhalation of a histamine aerosol (mg./kg.)	Spasmolytic activity on the isolated intestine of guinea-pig (Antergan = 1)
$-CH_2CH_2N(CH_3)_2$ (Antergan)	1 mg.	1
$-CH_2-CH(CH_3)-N(CH_3)_2$	25 mg.	=
$-CH_2CH_2CH_2N(CH_3)_2$	20 mg.	1/15

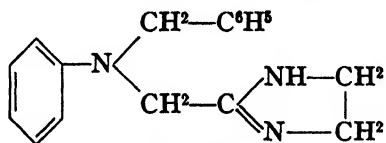
in the series of sympathomimetic and parasympathomimetic substances. The "optimum" is often represented by the  $\beta$ -amino-aliphatic chains, according to the rules laid down by Barger and Dale for adrenalin derivatives and by Hunt for cholines.

Lastly, let us mention the effect of substitutions made on the benzene nucleus. It is surprising to find that such alterations in the molecule



strongly interfere with the pharmacodynamic activity in the case of aniline derivatives, but have almost no effect in the phenolic ether series.

Antistine, or benzyl-N-methylimidazoline-aniline, belongs to the same chemical series which includes many active drugs such as Otrivine, Privine



Antistine

and Prisco<sup>28</sup> and was also prepared by Miescher, Urec, and Klarer. Its molecule is composed of a benzyl group linked to aniline. In the case of Antistine, however, the aliphatic amine of the Antergan molecule is replaced by a more complex base, namely the imidazoline nucleus. This substance has an antihistamine activity similar to that of Antergan. According to Meier and Bucher,<sup>29</sup> Antistine produces in guinea pigs the typical effects of the antihistamine drugs, but higher doses are necessary to counteract histamine intoxication. In our tests, the ratio between Antergan and Antistine activity was found to be about 10 to 1.

With the group of phenolic ethers and the group of aniline derivatives in which the oxygen bridge of the phenolic ethers has been converted into a nitrogen bridge, we have briefly examined the part played by the amino substituents, by the substitutions of the benzene nucleus, and by the transformations effected in the side chain. There remained for consideration the influence on antihistamine activity of the replacement of the benzene nucleus itself by other radicals. Toward this end, we investigated in 1944, together with Walthert, Fournel, and Horclois,<sup>30, 31</sup> the derivatives of aminopyridine and described the activity of the most active compound, namely, N-p-methoxybenzyl-N-dimethylaminoethyl- $\alpha$ -aminopyridine, or Neoantergan. Neoantergan is the most active of all hitherto known antihistamine agents. By subcutaneous injection it exerts a preventive action against histamine intoxication in doses of less than one-tenth of a milligram (FIGURE 2).

The experiments which led to the discovery of Neoantergan included more than 30 derivatives belonging to the same chemical series. This was reported upon in detail by Viaud<sup>27</sup> in 1946 and by me in 1947 at the International Congress for Chemistry in London.<sup>22</sup> In 1945, Mayer, Huttner,

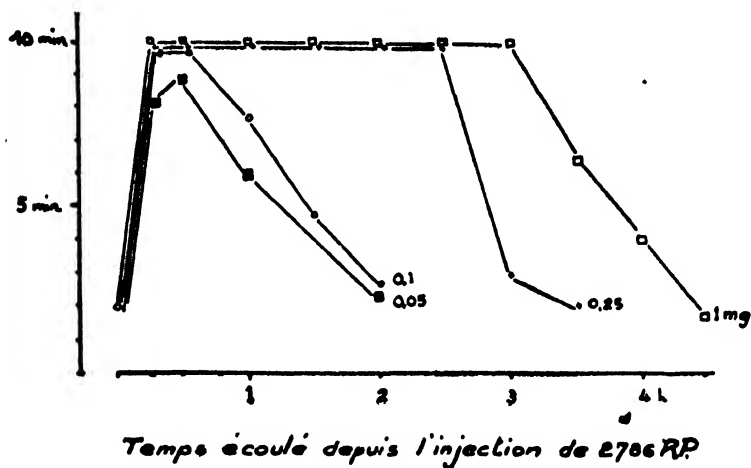
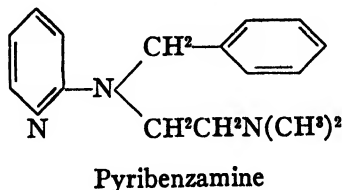
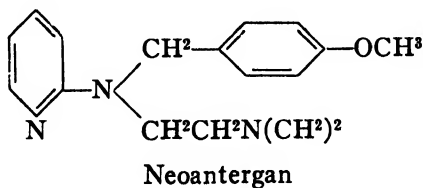
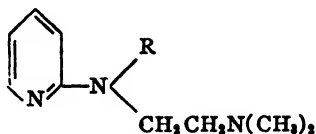


FIGURE 2. Antihistaminic activity of Neoantergan. Protection against bronchospasm induced in guinea pigs by inhalation of a histamine aerosol. Effects of increasing doses of Neoantergan injected subcutaneously into guinea pigs (mg./kg.). Ordinate: time of animal exposure in the inhalation box, before the appearance of severe asthma; Abcissa: time between injection of Neoantergan, and exposure to aerosol. The untreated animals show an average resistance of 2 minutes against the effect of histamine. Resistance is prolonged by a preventive injection of a single dose of Neoantergan (injected in time 0) (Bovet and Walther<sup>21</sup>).

and Scholz,<sup>23</sup> working in the same direction, recognized the activity of N-benzyl-N-dimethylethyl- $\alpha$ -amino-pyridine, which they introduced under the name of Pyribenzamine.<sup>24</sup> They have established the first elements of



the rules which in this group govern all relationships between pharmacodynamic activity and chemical structure. Pyribenzamine is about five times less active than Neoantergan in animals.



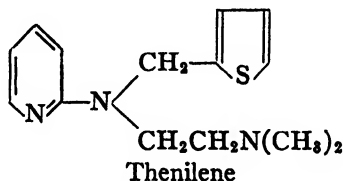
Dose of antihistamine drug permitting to effect prevention of bronchospasm caused by inhalation of a histamine aerosol (mg./kg.)

Spasmolytic activity on the isolated intestine of guinea-pig (Antergan = 1)

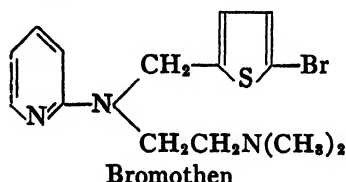
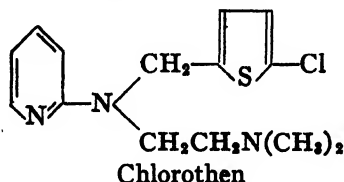
	0.5	1.3
(Pyribenzamine)		
	10	1/20
	no action	=
	0.1	1
(Neoantergan)		
	no action	1/80

If the methoxy group of the Neoantergan molecule is transferred from the para position of the benzene nucleus to the meta- or ortho-position, the activity of the compound decreases considerably.

The thiazole derivatives of these aminopyridine compounds have been

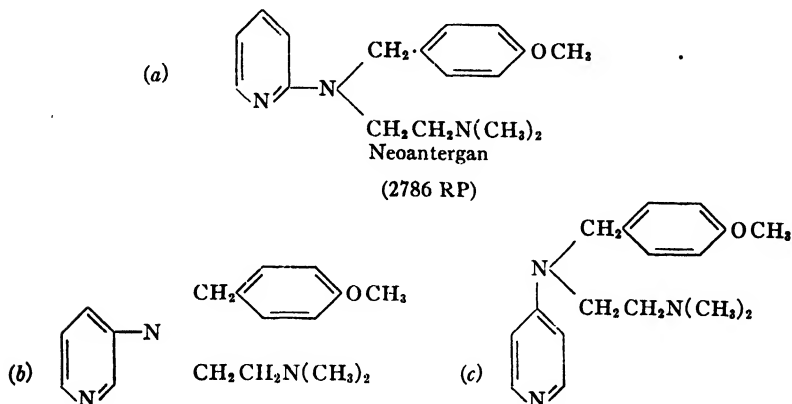


introduced<sup>35, 36</sup> under the name of Thenilene or Histadil, and the corresponding chloro- or bromoderivatives under the name of Chlorothen and Bromothen



respectively.<sup>36</sup>

It is somewhat surprising that the physiological properties of the pyridine derivatives vary, to a marked extent, with certain substitutions which do not produce the same variations in the case of the aniline derivatives of the Antergan group. Thus, the position of the amine in the pyridine nucleus plays an important role.



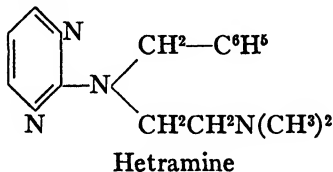
Dose of antihistamine drug permitting to obtain prevention of bronchospasm caused by inhalation of a histamine aerosol:

(a) 0, 1 mg.

(b) 10 mg.

(c) 50 mg.

Feinstone, Williams, and Rubin<sup>37</sup> have recently described the antihistaminic properties of a pyrimidine derivative, Hetramine. By comparison









with the antihistaminic substances already mentioned, this newcomer possesses only a low activity.

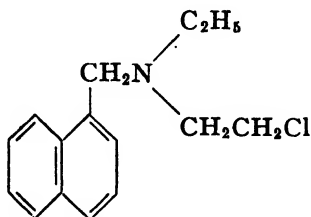
From the standpoint of their antihistamine properties, each of the various chemical series we have considered so far, namely, phenolic ethers, anilines, aminopyridines, have contributed toward definite progress. The protection afforded against histamine approached first 5, then 120, and later 300 lethal doses. The minimum active dose of antihistamine agent decreased from 20 mg. to 1 mg. and finally to 0.1 mg.

It is important to emphasize that Neoantergan is, like histamine, a triamine. This is possibly more than a mere coincidence and may indicate that we are dealing with a competitive phenomenon at the level of peripheral receptors.

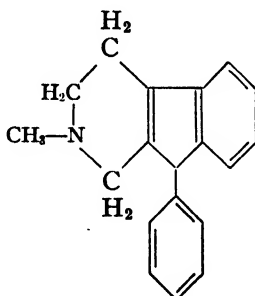
From the chemical standpoint, the four series of compounds already mentioned, namely, phenolic ethers, aminoethylanilines, phenylaminomethylimidazolines, and aminopyridines, have certain structural relationships to

	Toxicity mouse s.c. mg/kg	Protection against histamine shock		Protection against the toxic effect of a histamine aerosol		Protec. against anaphy- lactic shock
		number of doses	minimal active doses	mg/kg	duration 1 mg/kg 10 mg/kg	mg/kg
 <chem>CC(C)c1ccc(OCCN(CC)CC)cc1</chem>	929 F	500	5 d.	20 mg.		
 <chem>CCN(CC)c1ccccc1CCN(CC)CC</chem>	1571 F	750	10	10 mg.	10 min.	
 <chem>CN(C)CCc1ccccc1CNc2ccccc2</chem>	Anter- gan (2339 RP)	175	120	1	2h.	1 mg.
 <chem>CN(C)CCc1ccccn1CNc2ccccc2</chem>	Pyri- benza- mine	100	150	0.005	0.5 2h.	1 mg.
 <chem>CN(C)CCc1ccc(OC)cc1CNc2ccccn2</chem>	Neo- anter- gan (2786 RP)	150	300	0.0015	0.1 3-4h.	0.2 mg.
 <chem>CNCC(=N)Cc1ccccc1CNc2ccccc2</chem>	Antis- tine	150	30	10	1h	2-3 mg.

different groups of known sympathomimetic and sympatholytic compounds. One could add to this list, two other series, namely, alkylaromatic derivatives of  $\beta$ -chlorethylamine, recently described by Achenbach and Loew,<sup>38</sup>



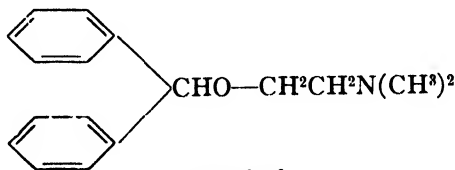
and phenyl tetrahydro-pyridindenes, the activity of which has been reported by Lehmann, Hagen, Berberow, and Roe.<sup>39</sup>



Thephorin

Two other groups not hitherto mentioned in this discussion, but which we shall now describe in some detail, are quite different from all previous groups in so far as they no longer are associated with any sympathetic activity. Rather, they are related to the spasmolytic agents and the acetylcholine antagonists. These groups are benzohydrylethers and phenothiazylethylamines.

Indeed, considering their structure, the ethers of benzyl alcohol, prepared by Rieveschl and Huber and investigated by Loew, Kaiser, and Moore,<sup>40-42</sup> are more closely connected with the spasmolytic agents of the Trasentine type than all preceding series. Benzohydrylethers, the most active of which is Benadryl, represent a very original contribution of American scientists to the antihistamine problem. The minimal active dose of Benadryl for the guinea pig is 5 mg. when given under the experimental conditions of

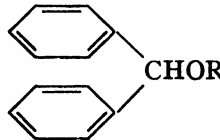


Benadryl



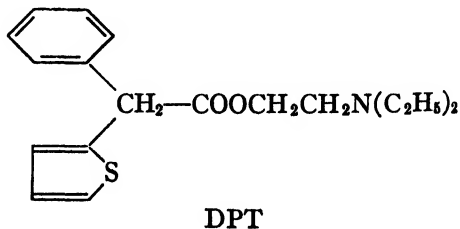
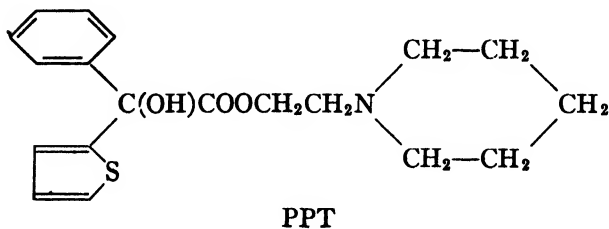
our own laboratories, whereas Loew found a threshold of activity of 1.5 mg. He has also found, however, for our products, a higher activity than that which we had established ourselves. Benadryl affords protection for the guinea pig against 75 toxic doses of histamine.

Loew and his co-workers have published some precise data concerning

	Protection of the guinea pig against the toxic effects of histamine aerosol.
	mg./kg.
R = CH <sup>2</sup> CH <sup>2</sup> NH <sup>2</sup>	25 mg.
CH <sup>2</sup> CH <sup>2</sup> NHCH <sup>3</sup>	6 "
CH <sup>2</sup> CH <sup>2</sup> N(CH <sup>3</sup> ) <sup>2</sup>	1.5 "
CH <sup>2</sup> CH <sup>2</sup> N(C <sup>2</sup> H <sup>5</sup> )	12.5 "
CH <sup>2</sup> CH <sup>2</sup> NC <sup>6</sup> H <sup>10</sup>	1.5 "

(according to Loew,  
Kaiser and Moore, 1945)

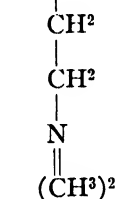
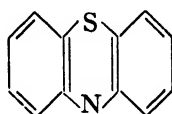
the relationship between the molecular structure and antihistamine activity in the Benadryl series. Several conclusions may be drawn from Loew's investigations, similar to those which we have formulated with regard to phenolic ethers. The most active side chain has two carbon atoms; tertiary amines have a higher activity than secondary or primary amines; and the dimethyl derivative is more active than the diethyl derivative. Loew<sup>41</sup> has also shown the high activity of the iodomethylate derivative of Benadryl. Other derivatives of the Trasentine group have been found active by Chen and Abreu,<sup>43</sup> such as:



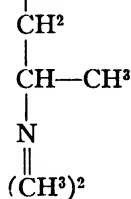
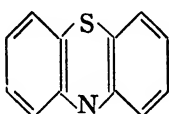
We shall terminate this long list of active compounds by referring to the phenothiazines, the study of which we had begun at a time when we were

engaged in a search not for antihistaminics, but for antimalarials. We are still investigating this group in our laboratory. The first results on the antihistaminic activity of these substances have been reported by Halpern.<sup>44</sup> The first synthesis of these phenothiazines was described in this country by Gilman,<sup>45</sup> but no pharmacologic investigation had been reported.

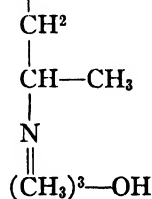
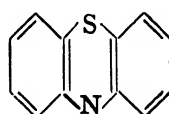
Although their threshold of activity is higher than that of Neoantergan, they are of special interest because of their long-lasting and powerful action. Phenothiazinylethyldimethylamine (3015 RP) is active in a dose of 0.5



3015 RP



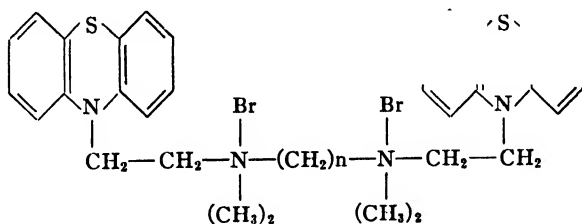
3277 RP



3554 RP

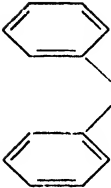
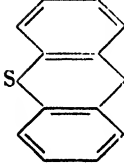

mg. and protects the guinea pig against 700 lethal doses of histamine. A corresponding isopropyl derivative, namely, 3255 RP, is still more active.

We are now investigating certain derivatives of the same group with a quaternary function and various other derivatives of high molecular weight which are better tolerated.<sup>32</sup>



n = 2  
n = 4  
n = 5 (3550 RP)  
n = 6  
n = 7

Protection of the guinea-pig against the toxic effect of a histamine aerosol		Protection secured against histamine shock
mg./kg.		number of doses
0.5	2-3 h.	400
0.5	6 h.	500
0.5	6 h.	700
1.0	5 h.	600
1.0	5 h.	400

	Toxicity mouse sc. mg./kg.	Protection against histamine shock		Protection against the toxic effect of a hista- mine aerosol		Protec. against anaphy- actic shock
		number of doses	minimal active doses	mg./kg.	duration mg. 10mg.	mg. kg.
 <p style="text-align: center;">BENADRYL</p> <p style="text-align: center;"><chem>CHO-CH2CH2N(CH3)2</chem></p>	150	75	0.06	5	—3h	1—2mg.
 <p style="text-align: center;">3277 RP</p> <p style="text-align: center;"><chem>N-CH2-CH(CH3)-N(CH3)2</chem></p>	225	1200	0.02	0.5	7h —	0.1mg.
 <p style="text-align: center;">3550 RP</p> <p style="text-align: center;"><chem>N-CH2-CH2-N(CH3)2</chem>  <math>\begin{array}{c} \text{OH} \\   \\ \text{CH}^2 \\   \\ \text{CH}^2 \\   \\ \text{CH}^2 \\   \\ \text{CH}^2 \\   \\ \text{CH}^2 \\   \\ \text{OH} \end{array}</math></p>	500	700	—	0.5	6h —	

Having thus reached the end of this enumeration, we shall now attempt to establish the characters common to the various series of antihistamine products. Actually, only a few functions are common to these different groups. In the first place, they all possess one or more strongly saturated tertiary or quaternary amines. Secondly, the amino group is frequently found in  $\beta$  position, attached to an aliphatic chain. Thirdly, an amino group or an oxygen atom forms a linkage between a strongly electropositive nucleus and a basic side chain.

In addition to these very general rules, there exists in each of the series considered a characteristic element which emphasizes its activity. The role of these elements still escapes us entirely, so that all investigations in this field have a highly empirical character. In the series of phenolic ethers, substituents on the benzene nucleus play a part which does not appear to have any significance in the case of the aniline compounds. Indeed, the  $\text{OCH}_3$  group has a great importance when attached to the benzyl group of Neoantergan but is of no importance if present in the benzyl group of Antergan. In the phenothiazine series, the branching of the side chain plays an important role, which is lacking in the other series. We want to stress once again the significant fact that antihistaminic activity could always be detected in those substances which are structurally related to adrenolytic or spasmolytic compounds.

In a general way, this chemical study leads to the conclusion that only in one instance have we been able to establish a certain structural relationship between the antihistaminic agents and histamine itself.

In spite of the chemical diversity of the various antihistaminic agents, there exists a great homogeneity in their pharmacodynamic properties. TABLE 1 illustrates the general activity of the various antihistaminics upon histamine effects.

I shall now report some quantitative data relevant to Neoantergan, which, as has been shown, is a particularly active product. The concentrations active on isolated organs are often smaller than those of histamine itself. Dews and Graham<sup>46</sup> have reported that, on the rabbit ear, which is particularly sensitive, this product exerts an activity in a dose of 1 thousandth of a microgram. By subcutaneous injection, Neoantergan manifests, in the intact animal, an antitoxic action in a dose of .03 mg. per kg. According to Winter,<sup>47</sup> the ratio for Neoantergan between the active dose in guinea pigs treated with a normally toxic dose of histamine and the  $\text{LD}_{50}$  is 1 to 68,000.

The protection against one or several lethal guinea-pig doses of histamine represents the most specific criterion so far employed. The definitely toxic dose of histamine for an unprotected animal injected intrajugularly varies between 0.4 and 0.9 mg./kg. The test method consists of injecting increasing doses of histamine successively into the vein of the guinea pig, protected by the subcutaneous injection of the drug. The results are given in a previous paper,<sup>9</sup> with reference to the toxic dose of histamine for normal animals.

As already indicated, this method has been subsequently utilized by Staub<sup>10</sup> (phenol and aniline derivatives); by Rosenthal and Brown<sup>13</sup> (929 F); by Burchell and Varco<sup>14</sup> (929 F and 1571 F); and by Halpern<sup>26</sup> (Antergan). Remarkable results have been obtained with Neoantergan, whose activity is noticeable at the (subcutaneous) dose of 0.1 mg./kg.<sup>30, 31</sup> (see TABLE 2).

The maximum protection assured by the compounds belonging to the different chemical series have been mentioned previously. Friedlaender, Feinberg, and Feinberg<sup>48</sup> have expressed the antihistaminic activity by the number of doses of histamine necessary to cause death in the guinea pig

TABLE I  
ACTION OF THE ANTIHISTAMINICS ON THE PHARMACODYNAMIC EFFECTS OF HISTAMINE

		<i>Effects on the capillary permeability</i>	
<i>Toxicity</i>	Guinea pig.....	protection (T, A, N, B, P)*	..... augmentation of the permeability
	Rabbit.....	protection (N)	..... Lewis's triple reaction..... antagonism (N, B)
	Dog.....	protection (N)	..... hemoconcentration..... antagonism (A, N, B, P), antagonism (T)
<i>Arterial pressure</i>	Mouse.....	no protection (N, P)	
	Rabbit.....	hypertension.....	antagonism (T, A, N)
	Rabbit.....	hypotension.....	weak antagonism (T, A, N), or without action (T, A, N)
<i>Cardiac effects</i>	Dog.....	hypotension.....	antagonism (T, A, N, B, P)
	Ca. t.....	hypotension.....	weak antagonism (N)
	Ca. t.....	unstable action (P)	
		<i>Effect on the bronchial muscles</i>	
<i>Vasomotor effects</i>	Guinea pig.....	isolated lung.....	bronchoconstriction..... antagonism (T, A, P)
	Guinea pig.....	lung <i>in situ</i> .....	bronchoconstriction..... antagonism (T, A, N)
	Rabbit.....	bronchoconstriction.....	antagonism (T, A, N)
<i>Effect on the stomach muscles</i>	Dog.....	bronchoconstriction.....	antagonism (A, N, P)
	Guinea pig.....	contraction.....	antagonism (T)
	Guinea pig.....	contraction.....	antagonism (T, A, N, B, P)
<i>Effect on the intestine muscles</i>	Rabbit.....	contraction.....	antagonism (A)
	Dog.....	contraction.....	antagonism (T, N, B, P)
<i>Effect on the uterus muscles</i>	Rat.....	contraction.....	without effect (A)
	Guinea pig.....	contraction.....	no antagonism or weak antagonism (T, A)
	Rabbit.....	contraction.....	antagonism (A, N)
<i>Lachrymal secretion</i>	Dog.....	contraction.....	antagonism (N, B, P)
	Cat.....	Hypersecretion.....	antagonism (P)
<i>Salivary secretion</i>	Cat.....	Hypersecretion.....	antagonism (P)
	Pigeon.....	Hypersecretion.....	without effect (A)
	Rat.....	Hypersecretion.....	without effect (N)
<i>Gastric secretion</i>	Dog.....	Hypersecretion.....	without effect, exceptional antagonism (T, A, B)
	Man.....	Hypersecretion.....	without effect or unconstant action (A, N, B, P)

\* T = Thymoxyzethyldiethylamine (929 F.); A = Antergan; N = Neointergan; B = Benadryl; P = Pyribenzamine.

previously protected by the subcutaneous injection of 3 mg./kg. of the drug. For the different products, they have found the following results:

*Antihistaminic activity<sup>43</sup>*

Benadryl.....	5
Antergan.....	6
Pyribenzamine.....	37
Neoantergan.....	125

With the strong dose of 20 mg./kg. of a derivative of thiodiphenylamine (3277 RP), one can succeed in partially protecting guinea pigs against 1200 toxic doses of histamine. However, animals die eventually from gastric ulcers and perforations.<sup>44</sup>

Winter<sup>47</sup> has developed a somewhat different test, which permits the determination of the dose capable of protecting 50 per cent of the animals.

TABLE 2  
ANTITOXIC ACTIVITY OF NEOANTERGAN AND ANTERGAN<sup>51</sup>

	<i>Dose injected subcutaneously— mg./kg.</i>	<i>Number of treated animals</i>	<i>Intravenous histamine</i>	
			<i>number of mini- mal lethal doses tolerated</i>	<i>total quantity of histamine received per kg. in one injection (mg.)</i>
Neoantergan	0.1	4	5 to 10	3 to 6
	0.25	2	15 to 20	9 to 12
	0.5	4	30	18
	1	11	75	45
	20	—	300	180
Antergan	1	2	3 to 5	1.8 to 3
	2.5	5	30	18
	5	5	40	24
	10	6	45	27
	20	—	120	72

In this way, considering the toxicity of the products for the mouse, Winter established a therapeutic index which appears to be very high for some derivatives (TABLE 3).

The effects caused by histamine in different species of animals cannot be superimposed. While spasmodic phenomena predominate in the guinea pig, vasomotor phenomena are relatively more important in the dog. Moreover, the lethal doses of histamine vary on a large scale from one species to another. By intravenous injection, nearly 0.5 mg./kg. of histamine is sufficient to kill the guinea pig, 2 to 20 mg./kg. will kill the rabbit and the dog, while 400 mg./kg. are required to kill the mouse.

As with these differences in the susceptibility to histamine, there also exist considerable differences in the reactions provoked by the antihistamine substances in various animal species. In a general way, the greater the susceptibility to histamine, the higher is the protection.

We have repeated on the rabbit and on the dog the same assays as per-

formed on the guinea pig.<sup>31</sup> For the rabbit, the protection assured by Neoantergan approaches 30 minimal lethal histamine doses; in dogs, the protection extends only to two and a half toxic histamine doses. In mice, it has been shown that not only did the antihistaminics confer no protection, but the toxicity of these antihistaminics was added to that of histamine. This phenomenon, formerly described for Neoantergan,<sup>31</sup> has been confirmed during the study of Pyribenzamine.<sup>49</sup>

TABLE 3  
ANTITOXIC DOSE, THERAPEUTIC INDEX, AND TOXICITY PECULIAR TO CERTAIN  
ANTIHISTAMINIC DRUGS\*

	<i>Antihistaminic effect—minimal active dose</i>	<i>Toxic dose D.L. mouse (i.p.)</i>	<i>Therapeutic index</i>
Hetramine.....	0.150	76	500
Benadryl.....	0.060	140	2,333
3277 R.P. ....	0.020	190	9,500
Pyribenzamine.....	0.003–0.005	68	19,400
Neoantergan.....	0.001–0.005	102	68,000

\* Data taken from Winter.<sup>47</sup>

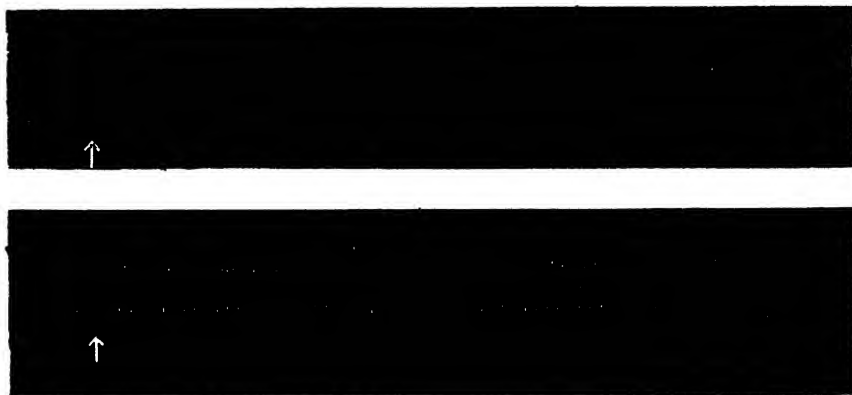


FIGURE 3. Action of thymoxyethyldiethylamine (929 F) on the histaminic bronchoconstriction of the guinea pig. Registration of the bronchial caliber *in situ* according to the technique of Cordier and Magne. *1st line*: action of 0.05 mg./kg. of histamine hydrochloride (i.v.). *2nd line*: action of the same dose of histamine in an animal previously treated with 5 mg./kg. of 929 F. intravenously (Staub<sup>10</sup>).

The protection of guinea pigs against one or more lethal doses of histamine and the prevention of histaminic asthma by antihistaminics given in the form of an "aerosol" represent a uniform and constant phenomenon, namely the suppression of bronchial spasm provoked by histamine. Experimentally, it may be observed by different methods.

According to the method of Cordier and Magne, which we used in our early work with Staub,<sup>10</sup> the guinea pig is placed in a closed box and air is introduced under a regular pressure directly into the trachea. The recording of the velocity of the air passing through the trachea after every inspira-

tion, or of the quantity of air penetrating the thoracic cavity, proves that histamine provokes an intense bronchoconstriction which does not occur after the animal has been treated with thymoxyethyl-diethylamine (929F) (FIGURE 3).

According to a technique of Konzett and Roessler, utilized by Halpern<sup>26</sup> in his study on Antergan, the volume of air introduced is constant, and the portion of the air which does not penetrate into the lungs in great quantities is recorded after every inspiration in a manometer. We have verified once again, with Walthert,<sup>31</sup> the utmost sensitivity of this method. Following the injection of 1 mg. of Neoantergan into the jugular vein of the guinea pig, the sensitivity of the guinea pig towards histamine is diminished by 50 per

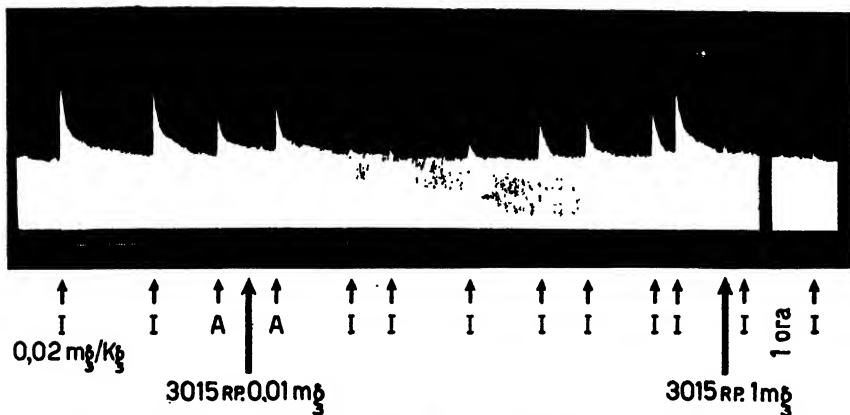


FIGURE 4. Activity of small doses of the antihistaminic drug, 3015 RP (phenylthiazinylethyldimethylamine), on the bronchospasm produced by intravenous injection of histamine in the guinea pig. The diminution of the bronchial caliber is characterized in this experiment (using the technique of Konzett and Roessler) by an increase of the height of the graph. It may be noticed that 3015 RP counteracts the bronchoconstricting effects of histamine (I), whereas it does not alter in any way the bronchoconstriction induced by acetylcholine (A). A small dose of 3015 (0.01 mg./kg.) produces transitory effects; the action is prolonged after a larger dose (1 mg.). (Bovet 1947<sup>1</sup>).

cent. After 1 mg./kg., a dose of histamine 30 times greater than the normal dose is needed to produce bronchoconstriction (FIGURE 4).

Using the plethysmography of the lung, as described by Jackson, Yonkman<sup>104</sup> has observed the antagonistic effect of Pyribenzamine on histaminic bronchoconstriction in the dog. With the isolated lung of the guinea pig, prepared according to the method of Sollmann, Staub has been able to demonstrate the antagonism towards histamine of the products, 929F and 1571 F,<sup>11</sup> and Yonkman that of Pyribenzamine,<sup>50</sup> thus demonstrating the peripheric nature of the protective effect.

Besides the bronchia, many other smooth muscular fibers of the intestines, stomach, uterus, and bladder respond with a change of tonus to the administration of histamine, usually presenting intense spasms to the administration of very minute doses. This is particularly evident with the intestinal muscles of the guinea pig, which react to a histamine concentration of the order of one to a billion.

The use of the isolated guinea-pig intestine constitutes the preferred



criterion for the demonstration of the effectiveness of various antihistaminic substances. They prevent the histamine spasm or bring about the relaxation of the contracted intestine. The effect of histamine at the concentration of  $10^{-7}$  (0.1 mg./l) on the guinea-pig gut is inhibited by the same concentration of 929 F or 1571F.<sup>9, 11</sup> With Antergan,<sup>26</sup> Neoantergan,<sup>31, 51</sup> and Pyribenzamine,<sup>33, 47</sup> the inhibiting dose is frequently smaller than the dose of histamine producing contraction (FIGURE 5).

On the other hand, the study of spasmolytic effects of the different compounds is excellently suited for the determination of the specificity of their properties. Antihistaminics oppose histaminic hypotension. This action was described for the first time by Rosenthal and Minard<sup>12</sup> while they were investigating thymoxyethyl-diethylamine. The hypotension provoked in

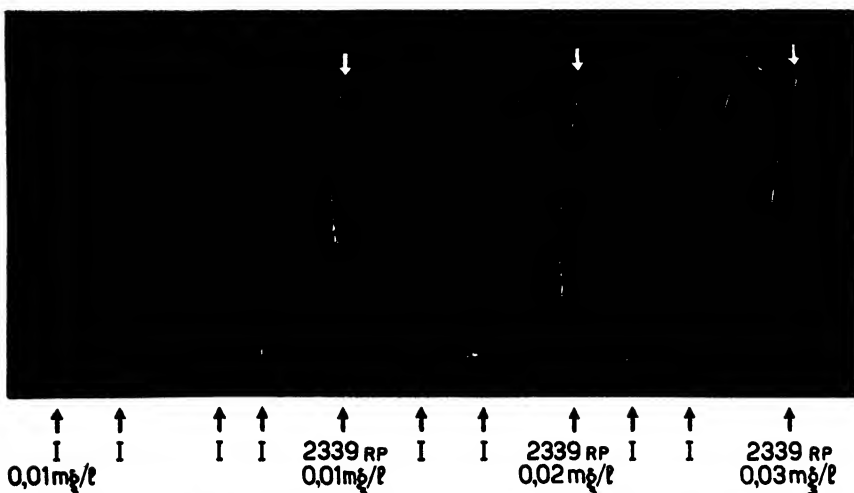


FIGURE 5. Action of Antergan (2339 R.P.) on the histaminic spasm of the isolated intestine of the guinea pig (I) (Bovet, 1947<sup>1</sup>).

ten dogs by an intravenous injection of 4 mg. of histamine averaged 40 mm. at the beginning of the experiment, while it was of no more than 10 mm. after administration of 929F. Observations of the same kind, but more demonstrative, have been made with Antergan,<sup>50</sup> Neoantergan,<sup>31, 52</sup> Pyribenzamine,<sup>50, 52</sup> and Benadryl<sup>41, 52, 53</sup> (FIGURES 6 and 7).

According to the observations of Sherrod,<sup>52</sup> Neoantergan, Pyribenzamine, and Benadryl have the same activity on the arterial pressure of the dog. Wells, Morris, Bull, and Dragstedt<sup>53</sup> have shown that the hypotensive effects produced in the dog by doses of 0.001–0.002 mg. of histamine are abolished by a dose of 3 mg./kg. of Benadryl. Examination of the responses indicate that a competitive antagonism similar to that between atropine and acetylcholine exists between Benadryl and histamine. The antagonism exerted by the antihistamine substances towards the tensional effects of histamine on the dog is impressive, although not so intense and long-lasting

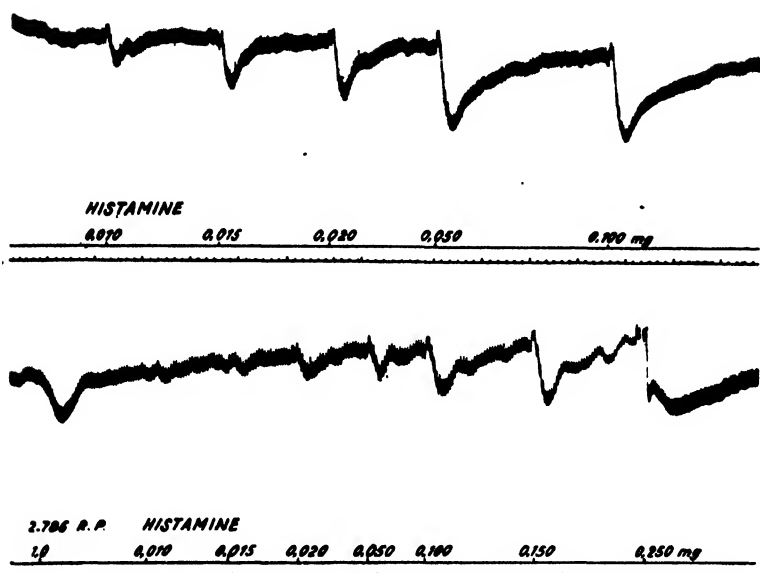


FIGURE 6. Antihistamine activity of Neoantergan. In a dog treated with 1 mg. of Neoantergan (2nd line), histamine has only slight tensional effects on the blood pressure (narcosis with chloralose; registration of arterial pressure; and intravenous injections of histamine chlorhydrate). Doses in mg./kg.; time: ten seconds (Bovet and Bovet-Nitti, 1948<sup>1</sup>)

Lapin A 37 : 3<sup>h</sup> 700  
 Histamine 0.2 mg intra-veineux  
 2786 RP 1 milligramme

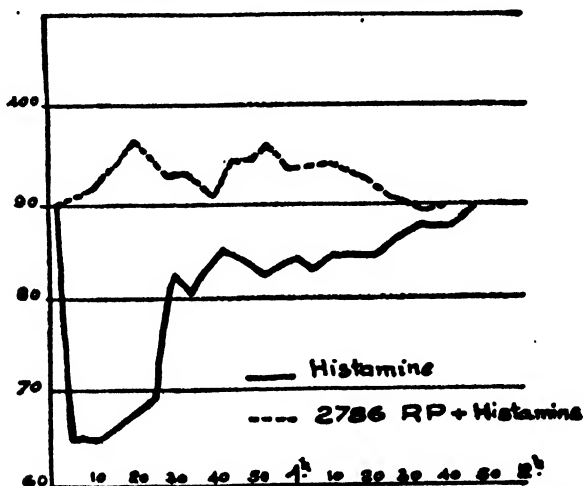


FIGURE 7. Effect of Neoantergan on the histaminic hypotension in the rabbit. Rabbit unanesthetized registration of the arterial tension according to Grant and Rothschild. The same animal served in the two tests performed with an interval of a week. The intravenous injection of 0.2 mg./kg. of histamine causes a fall of blood pressure in the normal animal (—) while, in the animal protected by a subcutaneous injection of 1 mg./kg. of Neoantergan (2786 RP) given a quarter of an hour previously (----), it remains without effect (Ramanamanjari, 1944, cit.<sup>11</sup>).

as that which one may observe, for instance, on the bronchia of the guinea pig.

It appears, from these investigations, that poisons of this type oppose not only the motoric effects but also the inhibiting effects of histamine. In this regard, the data on the antagonistic effects on the vasomotors of the intestines of various species are particularly instructive. We must mention here particularly the observation of Dews and Graham<sup>46</sup> on Neoantergan. They report that Neoantergan antagonizes not only the vasoconstricting effects produced by histamine on the blood vessels of the rabbit ear but also the vasodilatation effect in the paw of the dog and of the coronary arteries of the cat and the dog. Similar observations have been made on histaminic vasoconstriction of the coronaries of the rabbit,<sup>54</sup> and on vasodilatation of the nasal cavity<sup>51</sup> and splenic vasoconstriction in intact animals.<sup>46</sup>

Meier<sup>29</sup> has also noted that the vasoconstriction caused by perfusion of the paw of the rabbit with histamine in a relation of 1/1,000,000 instantly disappeared under the effect of Antistine in a dilution of 1/1,000,000. Neoantergan also opposes the tonic effect produced by histamine on the amplitude of the contractions of the isolated auricle of the rabbit.<sup>46</sup>

According to the tensional and vasomotor effects just mentioned, histamine produces, at the same time, a lesion of the capillaries characterized by an increase of their permeability. This process leads to a diffusion of plasma, leading to a diminution of the blood volume as indicated by an increase in the number of blood cells. In this way, histamine accelerates and facilitates the diffusion of the coloring substances introduced into the circulation and their fixation in the skin. The vasodilatation and the damage to the capillaries with their consequences, hypotension and hemoconcentration, constitute an acute phase of histamine intoxication.

Researches on the protection exerted by the antihistaminics in this field are numerous. Celice and Durel<sup>55</sup> and Parrot<sup>56</sup> have stated that, in man, the local susceptibility to histamine (Lewis's triple reaction) is diminished or suppressed by Antergan, and their findings have been frequently confirmed<sup>57</sup> and applied to other products such as Neoantergan,<sup>46</sup> Pyribenzamine,<sup>58</sup> and Benadryl.<sup>59</sup>

Antihistaminics do not oppose, in most cases, the gastric secretion produced by histamine. Any effect is usually secondary and probably due to the atropinic action the antihistaminics exert. The first observations on this subject have been made by the investigators who studied thymoxy-ethyldiethylamine (929F) and N-diethyl-aminoethyl-N-ethylaniline (1571F) on the dog: Loew and Chickering,<sup>17</sup> Burchell and Varco,<sup>16</sup> Bourque,<sup>18</sup> and Hallenbeck.<sup>19</sup> They noted that the inhibition of the gastric secretion, when it occurred, was not specific, since these substances also depressed the hypersecretion caused by pilocarpine<sup>18</sup> or mecholyl<sup>19</sup> in the same way they suppressed histaminic hypersecretion. In a general way, the antagonism appeared to be more obvious on the physiological response of the gastric secretion to food than to histamine.<sup>19</sup>

Similarly, Antergan does not modify the character of the gastric secretion. This has been verified by Gordonoff<sup>60</sup> on the dog and on the pigeon.

Also, Neoantergan does not modify the gastric secretion in the rat following the injection of histamine<sup>81</sup> (FIGURE 8). The newer antihistaminics, although much more active against histamine, have no more consistent effect on the gastric secretion. The results with Benadryl<sup>59, 62</sup> and Antergan<sup>63</sup> are still irregular, in both the laboratory<sup>41, 61</sup> and the clinic.

The study of the antihistaminic agents has furnished interesting observations on the physiological function and physiopathologic effects of histamine. From the beginning of our studies, we were impressed by the absence of any specific activity of the antihistaminic substances on the normal organism.

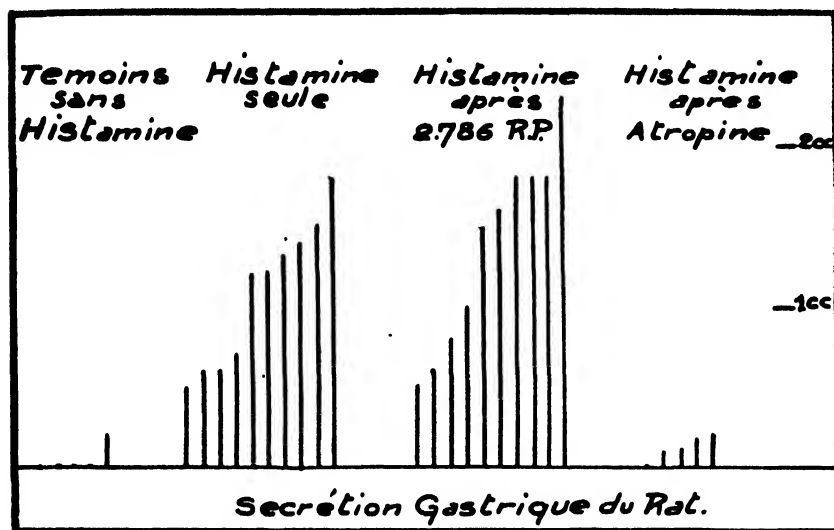


FIGURE 8. Neoantergan does not counteract the gastric hypersecretion produced by histamine in the rat, while atropine inhibits it. Tests on the rat: histamine 50 mg./kg. subcutaneously; Neoantergan 50 mg./kg. subcutaneously; atropine 200 mg./kg. subcutaneously. Each column corresponds to the quantity of gastric juice present in the stomach of the rat 1½ hours after the administration of the histamine. Results: (1) increase in the gastric secretion after histamine; (2) the preventive administration of Neoantergan (2786 RP) 30 minutes before histamine administration did not prevent histamine hypersecretion; (3) atropine, injected under the same conditions, prevented almost entirely the histaminic gastric hypersecretion (Bovet and Walther<sup>81</sup>).

In normal animals, atropine produces characteristic symptoms which must be interpreted as nullification of normal acetylcholine functions. The fact that Neoantergan does not produce any specific untoward effect in normal animals could be interpreted as a proof that histamine has no important physiological function. The few minor disturbances following injection of Neoantergan into a healthy individual are difficult to explain on the basis of a counteraction of important histamine functions. It is possible, however, to save the life of a guinea pig after administration of a lethal dose of Neoantergan by an injection of histamine.<sup>54</sup> This fact demonstrates, for the first time perhaps, that, to use a familiar expression, histamine "serves for something."

Experiments made with synthetic antihistamine drugs constitute, in both the laboratory and the clinic, a striking demonstration of the hypothe-

sis that histamine represents a predominating factor in causing anaphylactic and allergic troubles. The results of different authors on the antihistamine substances indicate a close parallelism between the antihistaminic properties and the antianaphylactic properties of synthetic derivatives. The use of antihistamine drugs permits us, today, to establish the boundaries of that large domain of allergy which depends in a particular way on the reactions provoked by histamine.

The antiallergic activity of the antihistamine substances manifests itself distinctly during the following syndromes:

- (a) anaphylactic shock of the guinea pig;
- (b) anaphylactic shock of the rabbit;
- (c) anaphylactic shock of the dog with humoral, cardiovascular, digestive, respiratory, and nervous involvement;
- (d) Schultz-Dale reaction, provoked by the addition of allergen to the isolated organs of a sensitized animal;
- (e) cutaneous allergy;
- (f) allergic reactions to chemical agents;
- (g) Sanarelli-Shwartzman reaction of local sensitization; and
- (h) experimental nephritis of Matsugi (inverted allergy).

The first observations on the treatment of anaphylactic conditions with antihistamine drugs have been made on the guinea pig, the ideal animal for this purpose. Animals sensitized with one or more subcutaneous or intraperitoneal injections of heterogeneous albumin (ovalbumin, serum) are, after three weeks, abnormally sensitive to the intravenous injection of a minute dose of protein and present the Arthus phenomenon.

With Staub,<sup>11</sup> we have noted that the administration of 929F (40 mg./kg. s.c.), thirty minutes before the re-injection of appreciable amounts of serum, exerts a protective action. This observation has been repeated several times (FIGURE 9). The type of antigen and the method of administration have been changed, but the results are of the same order, protection being afforded in a regular manner.

Rosenthal and Brown's experiments<sup>12</sup> were on guinea pigs sensitized three weeks prior to treatment with an intraperitoneal injection of 20 mg. of ovalbumin. They showed that, in untreated animals, a dose of 0.04 cc. of antigen provoked anaphylactic shock, while animals protected by 929F (40 mg./kg.) presented no reaction, even after receiving a 2.5 times greater dose of antigen.

We were also able to obtain protection with 0.1 mg./kg. of Neoantergan<sup>20, 21</sup> when the shocking dose was administered by inhalation of an aerosol of ovalbumin. During one experiment, an inhalation of aerosol containing 10 per cent ovalbumin was administered to several young sensitized guinea pigs. In unprotected animals, severe and generally irreversible reactions appeared rapidly, while all treated animals survived and showed, if any, only moderate anaphylactic symptoms (TABLE 4).

Several antihistaminic drugs have been employed in this test. Staub,<sup>11</sup> Wilcox and Seegal,<sup>22</sup> and Loew<sup>24</sup> have described the activity of 1571F; Halpern<sup>25</sup> has found that Antergan protects in a dose of 20 mg./kg., which

seems to be greater than the therapeutic dose; Loew, Kaiser, and Anderson,<sup>66</sup> Friedlaender, Feinberg, and Feinberg,<sup>66</sup> and Selle<sup>67</sup> have recognized the

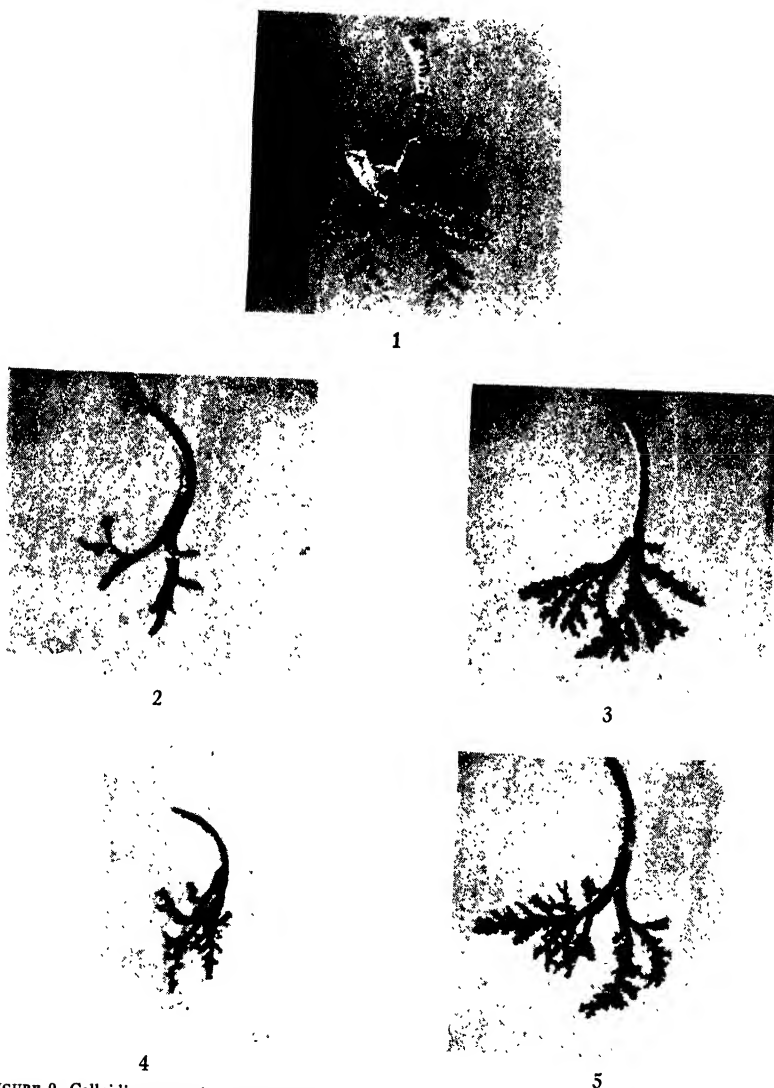


FIGURE 9. Celloidin casts of guinea-pig lungs according to Hanzlick. (1) Control, normal lung. (2) Lung of a guinea pig intoxicated by one lethal dose of histamine (1 mg./kg.); incomplete filling due to bronchial obstruction. (3) Protection given by an injection of Neoantergan to the guinea pig intoxicated by the same dose of histamine. (4) Incomplete filling due to the bronchial obstruction in anaphylactic shock. (5) Protection afforded by Neoantergan against a shocking dose of antigen in a sensitized animal (Bovet, 1947).

action of Benadryl; Mayer, Huttner, and Scholz,<sup>33</sup> Baer and Sulzberger,<sup>68</sup> and Friedlaender<sup>66</sup> that of Pyribenzamine; and similar properties have

been recorded with Antistine,<sup>29</sup> Hetramine,<sup>37</sup> a derivative of thiodiphenylamine,<sup>44</sup> and  $\alpha$ -naphthylmethyl- $\beta$ -chlorethylamine.<sup>38</sup>

From data given by Loew<sup>66</sup> we have listed the values (TABLE 5) which he obtained in an experiment on passive sensitization, during which the guinea pigs received, successively, a preparatory injection of 0.5 cc. of antiovalbumin serum and a provocative injection of a 0.2 per cent solution of ovalbumin.

In a general way, it is interesting to note that the antihistaminic and anti-allergic properties of different products parallel each other closely. This

TABLE 4  
ACTION OF NEOANTERGAN ON THE ANAPHYLACTIC SHOCK PROVOKED IN GUINEA PIGS  
BY INHALATION OF OVALBUMIN AEROSOL<sup>31</sup>

<i>Dose of 2786 RP mg./kg. s.c.</i>	<i>Number of animals</i>	<i>Average time of exposure to aerosol</i>	<i>Results</i>
0 mg. (controls)	7	3½ minutes	3 sudden deaths 2 violent and reversible shocks 1 pruritus without respiratory difficulties
0.1 mg.	3	10 minutes	respiratory difficulties; not serious
1.0 mg.	6	10 minutes	3 guinea pigs—no symptoms 3 others with dyspnea after 2, 6, and 9 minutes

TABLE 5  
ACTION OF 1571 F. AND BENADRYL ON PASSIVE ANAPHYLAXIS ON THE GUINEA PIG

	<i>Dose i.p. mg./kg.</i>	<i>Number of animals</i>	<i>Intensity of shock</i>						<i>Mortality %</i>
			0	+	++	+++	++++	dead	
Untreated animals	—	45	2	13	6	1	—	23	51
1571 F	3	13	1	1	7	2	—	2	15
Benadryl	3	14	—	5	4	4	1	1	7.1
	12	15	2	6	6	—	—	1	6.6

mutual relation is, nevertheless, not absolute. Friedlaender, Feinberg, and Feinberg<sup>66</sup> have compared, in guinea pigs, the antianaphylactic effects of Pyribenzamine and Benadryl. They noted that both drugs, given in identical doses and under the same conditions, afford the same degree of protection, although their antihistaminic properties are very different. The observations made with other species of animals prove that the effectiveness of the various drugs depends upon the nature of the agents producing the shock, a phenomenon similar to that observed with histamine.

The antianaphylactic activity of antihistaminic drugs in rabbits has been studied by Pasteur Vallery-Radot, Bovet, Mauric, and Holzer.<sup>69</sup> Diethylaminomethylbenzodioxan and diethylaminoethoxydiphenyl (1262F) protect

rabbits regularly against histaminic shock, whereas the action of piperidino-methylbenzodioxan (993F) and thymoxyethyl-diethylamine (929F) is less consistent. Campbell and coworkers have noted only negative results with Benadryl.<sup>70</sup> Halpern<sup>26</sup> has made a detailed study of the action of N-ethyl-N-dimethylaminoethylaniline (2325 RP) and of Antergan in the anaphylactic shock in dogs. His results have been confirmed by us<sup>31</sup> with Neoantergan and by Yonkman, Hays, and Rennick<sup>71</sup> with Pyribenzamine.

The anaphylactic syndrome of the dog is characterized by disorders of the blood and blood vessels (hypotension, vasodilatation, and hemoconcentration due to the transudation of plasma through the capillaries), by disorders of the respiration (dyspnea caused by bronchospasm), by the reaction of smooth muscles (intestinal colics, diarrhea, vomiting, and miction), and by nervous disorders (algidity, torpor, hypothermia, and ataxia). The antihistaminic 2325 RP, injected into the dog at a dose of 10 mg./kg. a few minutes after the onset of shock, suppressed the intensity of the manifestations in 60 per cent of the tests, whereas Antergan injected similarly was active in all of the 6 tests.

In some experiments with Neoantergan, the protection was complete even with a dose of 1 mg./kg. (FIGURES 10 and 11). Benadryl affords equal protection against anaphylactic shock in the dog. Wells, Morris, and Dragstedt<sup>72</sup> found no mortality in a group of 22 dogs sensitized with horse serum, while 9 animals died in a group of 26 untreated animals (34.6 per cent). Mayer and Brousseau<sup>73</sup> tried to obtain, with antihistaminics, protection of the mouse against anaphylactic shock provoked by an intravenous reinjection of undiluted horse serum into sensitized animals. They observed a mortality of 89 per cent in the untreated animals and 42 to 70 per cent among the animals previously treated with Pyribenzamine. In no case, however, have mice been protected against the characteristic shock syndrome. Neither Antergan<sup>74</sup> nor Antistine<sup>75</sup> inhibit the formation of antibodies in the animal.

The protection conferred by antihistaminics against anaphylactic shock in the guinea pig extends to a great number of other manifestations of the allergic state. This is particularly true for the so-called "Schultz-Dale reaction." Results of this nature, with 929F, have been published in collaboration with Staub.<sup>10</sup> A portion of the small intestine from a sensitized animal contracts in the presence of the antigen, whereas that of a normal animal does not. These allergic reactions obtained outside the organism present the same specificity and sensitivity as that of the anaphylactic reaction of the whole organism. Introduced at a dilution of 1/2,000,000 into the bath liquid in which a section of the isolated organ of a sensitized guinea pig was immersed, 929F and 1571F inhibited the contraction caused by the antigen in the same way they inhibited the effect of histamine. Rosenthal and Brown<sup>18</sup> confirmed these results while studying the reactions of the guinea-pig uterus sensitized to ovalbumin. Other positive results were obtained by Meier and Bucher.<sup>29</sup>

A similar reaction can be provoked by the addition of allergen to the perfusion liquid of an isolated lung from a sensitized animal. Ellis<sup>76</sup> observed



that Benadryl afforded a definite protection, while in other assays, with Pyribenzamine,<sup>77</sup> negative results were obtained, probably due to differences in the intensity of the anaphylactic sensitization.

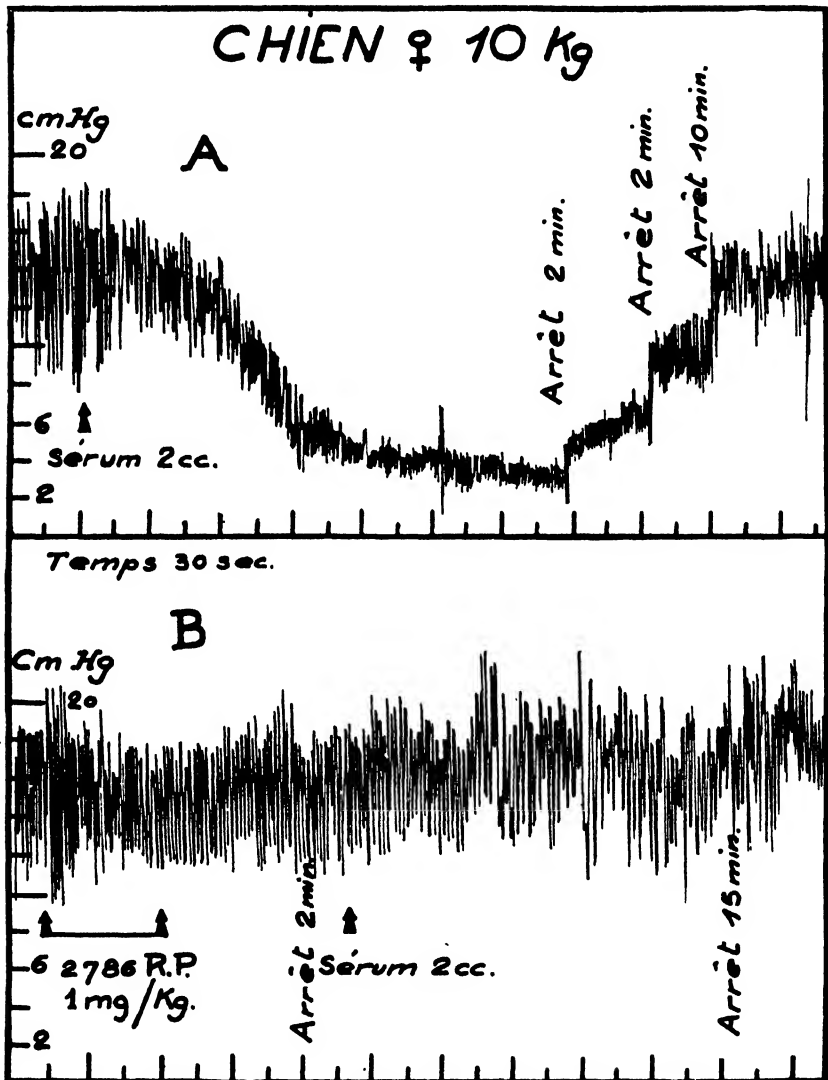


FIGURE 10. Antianaphylactic action of Neoantergan on the dog. Dog: 10 kg. The preventive injection of Neoantergan gave complete protection against shock. Twenty-five days after sensitization by injection of 0.25 cc. i.v. and 0.75 cc. i.m. ox serum, the dog received intravenously a provocative injection of 2 cc. of serum and presented characteristic shock with abdominal spasm, emission of urine, and hypotension. A reinjection of the antigen performed three days later, after a preventive dose of 1 mg./kg. of Neoantergan (2786 RP) had been given, did not produce any anaphylactic reaction (Bovet and Walther<sup>41</sup>).

Cutaneous allergy represents another field in which synthetic antihistaminics exert a characteristic action. Certain observations made in hu-

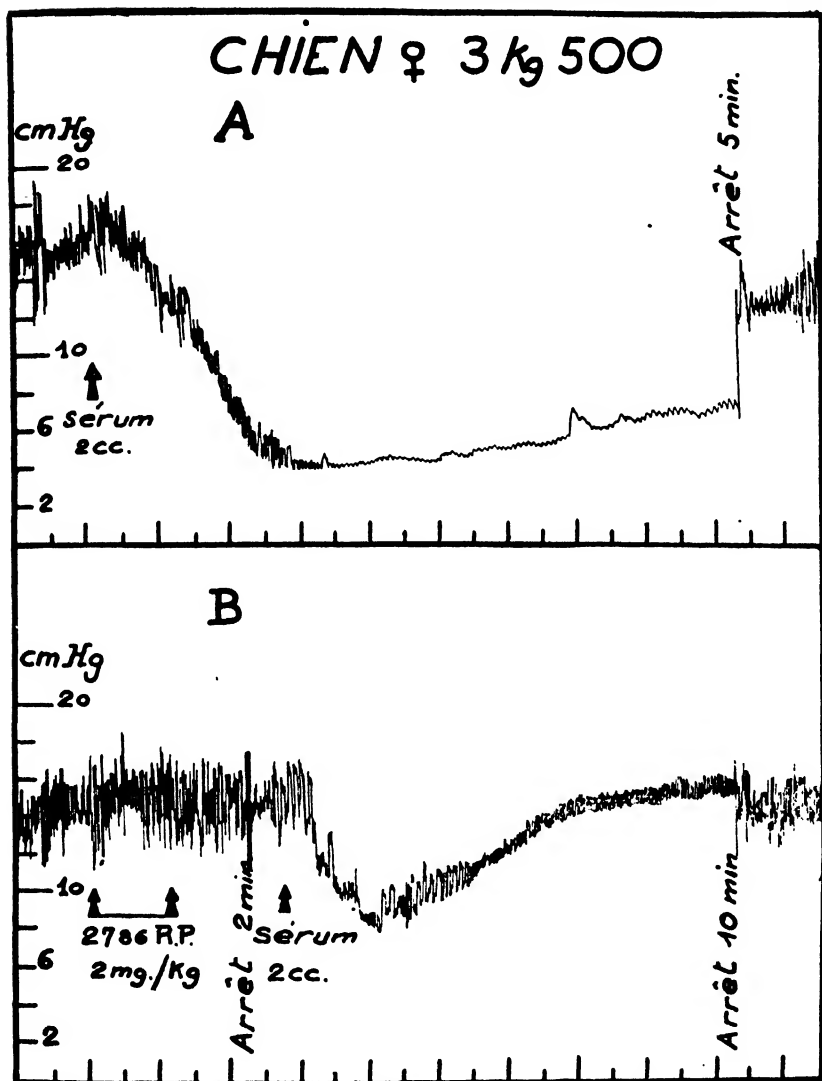


FIGURE 11. Antianaphylactic action of Neoantergan on the dog (continuation). Dog: 3.5 kg. The preventive injection of Neoantergan considerably attenuates the manifestation of an intense anaphylactic shock. 25 days after sensitization by an injection of horse serum (1 cc. i.v. and 1 cc. i.m.), the dog received intravenously a provocative injection of 2 cc. of the same serum, whereupon it presented severe shock with diarrhea, emission of urine, vomiting, hypotension, and cardiac involvement, followed by a period during which the pulse was hardly perceptible. The number of red cells rose from 5,630,000 to 7,920,000, corresponding to a hemoconcentration of 40 per cent. A second test, performed six days later, clearly showed the protection afforded by Neoantergan when administered a few minutes before the provocative injection of serum. The shock was extremely slight in the protected animal, producing only slight hypotension and no other troubles. The number of the red cells remained unchanged (4,520,000 to 4,760,000) (Bovet and Walther<sup>24</sup>).

mans have experimental significance, namely, those relative to the reactions produced by the intradermal injection of antigens in sensitive subjects and to the protection produced by the antihistaminics. The first observations

of Serafini<sup>78</sup> were confirmed by Friedlaender and Feinberg<sup>79</sup> (Antergan), by Harley<sup>80</sup> (Benadryl), and by Arbesman<sup>81</sup> (Pyribenzamine).

It is possible to reproduce, even in guinea pigs, other typically anaphylactic manifestations which represent cutaneous reactions to the allergen. The activity of antihistaminics against these manifestations is very constant. Mayer<sup>82</sup> points out their regression 15 to 35 minutes after a subcutaneous injection of Pyribenzamine (10 to 25 mg./kg.), while the symptoms reappear when the effect of drug has subsided, namely, 2 to 5 hours after the treatment.

It is apparent from the experiments of Mayer<sup>82</sup> that the evolution of contact dermatitis, which is much less susceptible to the effect of antihistaminics than anaphylactic reactions of the guinea pig, can nevertheless be influenced to some extent. Pyribenzamine attenuates and retards the development of dermatitis caused by para-phenylenediamine or dinitrochlorobenzene in the sensitized guinea pig. Histological examination reveals that the inflammatory reactions, edema, spongiosis, and parakeratosis, do not develop in the treated guinea pig to the same degree as in the untreated animal.<sup>83</sup>

Another aspect of the allergic reaction is the phenomenon of Sanarelli-Shwartzman, namely, the vascular hemorrhagic reaction produced at the level of the dermis. The experiments we shall now mention<sup>81</sup> indicate that the antiallergic activity of the antihistaminic substances, although not so consistent on the cutis as on the smooth muscle contraction, is evident (FIGURE 12).

According to the classic technique, rabbits, sensitized locally by intradermal injection, received, 24 hours later, an intravenous injection of allergen. In the areas where the intradermic infiltrations had been produced, some local reactions were now appearing. Clearly visible one hour later, the large ecchymosis and the edemas reached their maximum nearly 6 hours after the injection. The results of the test are given in TABLE 6. All untreated animals presented moderate to strong injury, while 65 per cent of the treated animals showed only slight or no reactions. Boquet<sup>84</sup> has observed negative results in similar tests with thymoxyethyl-diethylamine (929F) and Antergan. In our tests, these two products have shown activity, although it was inferior to that of Neoantergan.

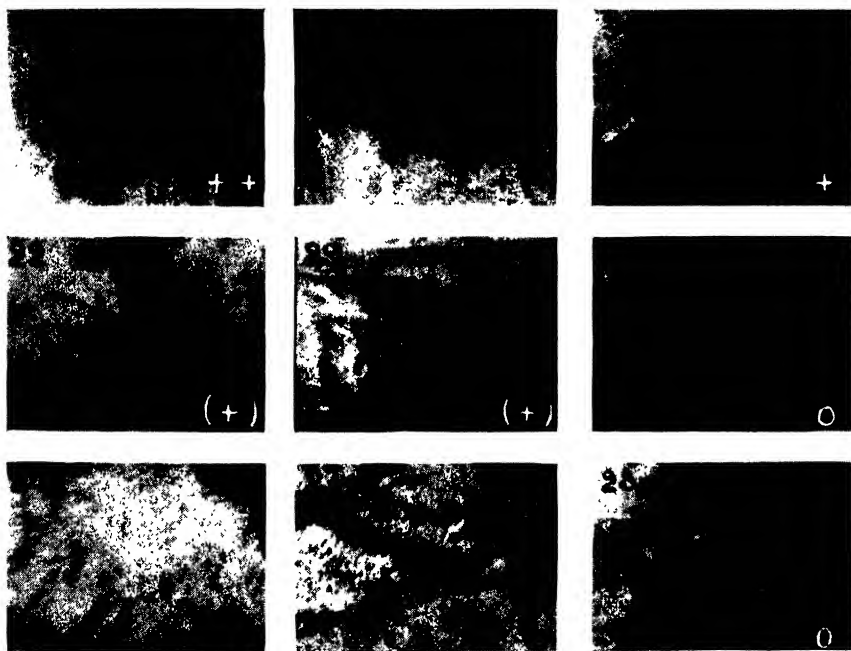
It has already been stated that, apart from anaphylactic reactions, histamine is liberated during various toxic injuries. The antagonistic effects of the antihistaminic substances in these cases permit us to elucidate the role of histamine. This is a relatively new field. The first observations in this direction were made in experiments by Sohier (cit.<sup>11</sup>) on the effect of 929F on the general disorders caused by intoxication with dichlorethylsulphide. Halpern<sup>26</sup> has demonstrated the protection against the peptone shock by antihistaminics.

Although the intravenous injection of trypsin in the dog causes symptoms similar to those observed in anaphylactic shock, Benadryl was without effect.<sup>72</sup> Only a slight attenuation of the hypotension was noted. It is not likely that, in this case, the increase in histamine blood level is important enough to account for severity of the reactions observed.

Another example is found in the experiments of Deschiens.<sup>85</sup> A toxin has been isolated from nematodes of the *Ascaris* type, which, according to Rocha e Silva and Deschiens, probably acts by liberating histamine. This observation is corroborated by the fact that Deschiens was able to demonstrate the protective action of Neoantergan against this toxin.



FIGURE 12. Action of Neoantergan on the hemorrhagic reactions of the Sanarelli-Shwartzman phenomenon. The photographs were taken 48 hours after the intravenous provocative injection. Top: 3 control animals; edema and more or less profound ulcerations. Bottom: 9 rabbits subjected to preventive and curative treatment by Neoantergan. The animals received 4 injections of Neoantergan at intervals of 3 hours; the first, 30 minutes before the provocative injection. (Total dose: 3.5 to 7.5 mg./kg.) In two rabbits, the reactions were no stronger than those occurring in the least reactive among the controls; three rabbits presented only insignificant lesions; and the epidermis of the remaining four was unaltered. The signs, 0, (+), +, and +++++ refer to the degree of the skin lesions observed (Bovet and Walther<sup>81</sup>).



In regard to the effects of drastic purgatives (castor oil, elaterium, globularia, and colocynth), Erspamer and Paolini<sup>86</sup> observed that antihistaminic substances effectively counteract the action of these poisons on the rat. The authors have implicated histamine in the reaction produced by these products. Similar observations concern the antagonism manifested by

antihistaminic substances against edema and hyperemia induced by dinoline.<sup>87, 88</sup>

Thus, experiments with antihistaminic compounds in animals have revealed widely divergent activities, such as tensional effects, effects on capillary permeability, spasmolytic actions, effects on secretions, and, finally, prevention of anatomo-pathological lesions.

It is not my intention to discuss the therapeutic aspects of antihistaminic compounds. They shall be dealt with thoroughly by others more familiar with this subject. I wish only to emphasize that, from the very beginning, when Antergan was first investigated in France in 1942, the same great diversity of activities was encountered in clinical patients as observed in experimental animals.<sup>89</sup> Clinicians now have at their disposal Benadryl<sup>90</sup> and Pyribenzamine<sup>91</sup> in the United States and Antistine<sup>92</sup> in Switzerland. We shall enumerate only the principal manifestations studied during the clinical experimentation, already representing a wealth of information on the physiopathologic role of histamine.

TABLE 6  
ACTION OF NEOANTERGAN ON THE PHENOMENON OF SANARELLI-SHWARTZMAN<sup>91</sup>

	Number of animals	Local reactions			
		none 0	weak +	medium ++	strong +++ and ++++
Untreated animals.....	10	"	"	"	5
Protection by Neoantergan*.....	20	4	9	4	3
					5
					-

\* Four subcutaneous injections within 24 hours represent a dose of 7.5 mg./kg. of 2786 R.P. The first injection was made 30 minutes after the intravenous challenge and two other injections three and six hours later, respectively.

Among all allergic affections, the pathogen role of histamine appeared, *a priori*, to be most evident in urticaria, the allergic wheal in acute urticaria presenting a close analogy with the cutaneous histamine wheal. Coinciding with the onset of the urticarial crisis, an increase in histaminemia is generally found. As a matter of fact, the high therapeutic efficacy of antihistaminic substances in the various forms of urticaria constitutes an additional proof of its histaminic nature. In the various forms of allergic urticaria, regardless of their origin, whether digestive or cryptogenic, a satisfactory effect of the treatment and sometimes an almost immediate cessation of the pruritus has been reported.

Antihistaminics act in a remarkable way in the majority of cases of serum disease and dermatographism. Besides urticaria, the antihistaminics are active in a multitude of dermatologic affections, particularly in Quincke's edema, in the allergic accidents during chemotherapy, and in various forms of pruritus.

The use of antihistaminics was also suggested in asthma, which, according to Widál, is the model of allergic manifestations, especially since the results obtained in the prevention of experimental histaminic bronchospasm or

allergic bronchospasm were quite remarkable. In human therapy, however, in spite of several very spectacular successes, numerous cases of doubtful or uncertain effects have been reported. It can be said that, depending on the product, approximately 15 to 50 per cent of the results are good or very good. At any rate, allowance must be made for the incidence of a large number of nonhistaminic asthma cases due to bronchopulmonary lesions or circulatory disorders, which are secondary phenomena and are brought about without intervention of histamine. Other affections influenced by antihistaminic treatment are hay fever, spasmodic rhinitis, and allergic conjunctivitis.

Before concluding, permit me to add a short remark. At several points in this paper, speaking of one or another chemical series, I have mentioned that certain chemical syntheses were attempted with the purpose of arriving at new sympatholytic, oxytoxic, spasmolytic, or antimalarial agents. Today, the impetus given by the study of the antihistaminic substances far exceeds the original scope of investigations. We now hope to find new physiological properties in those substances which originally were synthesized in our search for antihistaminics. Over many a circuitous route we have noted that certain phenothiazines are not only antihistaminic but also active in Parkinson's disease. With similar intention, we have resumed the study of phenolic ethers, this time seeking a new synthetic curare.

Thus, on each of the squares of that immense checkered table to which I alluded at the beginning of the paper, the pharmacologist throws his die and entertains the hope of its falling on a winning number.

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# THE ACTIVITY OF PYRIBENZAMINE AND RELATED COMPOUNDS WITH SPECIAL REFERENCE TO THEIR MODE OF ACTION

By R. L. Mayer

*Research Department, Ciba Pharmaceutical Products, Inc., Summit, New Jersey*

The anti-allergic properties of antihistaminic substances depend to a great extent upon the character, site, and state of the allergic manifestation against which they are employed. We know today with sufficient certitude that the present-day antihistaminics favorably influence hay fever or acute urticaria in 75 to 95 per cent of the treated cases and are much less effective in eczema or contact dermatitis. The reasons for these differences are not well known, but, since the outstanding activity of Pyribenzamine and related substances is their antihistaminic power, the differences in their anti-hay-fever and antidermatitic activities are attributed to the nature of the allergotoxin involved. Everybody supposes that therapeutic efficacy can be expected only in cases where the allergic manifestations result from an intoxication with liberated histamine.

This presentation will discuss whether this reasoning is correct under all circumstances and whether the antihistaminic activity explains the totality of the antiallergic effect. To this end, we shall compare the various pharmacodynamic and antiallergic activities of Pyribenzamine and certain other antihistaminic substances.

This comparison is restricted to the derivatives of ethylenediamine, amino ethanol, and imidazoline, namely, Pyribenzamine, Neoantergan, Benadryl, Antistine, and related compounds. According to our present knowledge, these substances act by the same physiologic mechanism, and the results obtained with one of them should apply to the entire series.

*Pharmacodynamics: The Antihistaminic Activity.* The principal point of attack of these substances is histamine (that is, in so far as therapeutic importance and degree or specificity of action are concerned).

Although in the preceding papers the pharmacodynamics of histamine has been thoroughly discussed, we shall, nevertheless, briefly summarize some of the most important activities. Histamine has a great number of physiological and pharmacological properties. It excites smooth muscles in many organs and stimulates the activity of various glands and nerve end-organs which perceive pain and other sensations. On the other hand, it paralyzes certain organ systems, including the capillaries and probably the lymphatics, and so forth.

The following histamine effects are counteracted by Pyribenzamine: <sup>1-5</sup>  
(1) the contraction of smooth muscles of the small intestines, the colon, uterus, the bronchi in guinea pigs and rabbits, and the nictitating membrane of cats; (2) the histamine-induced salivation and lachrymation in cats; (3) the dilatation of capillaries and wheal formation following the local injection, or the iontophoretic application, of histamine in animals and humans; (4) the hypotension which follows the intravenous injection of histamine.

Histamine-induced gastric secretion and hyperchlorhydria are practically unaffected by all known antihistaminics.

In many instances, the antihistaminic activity of Pyribenzamine or related substances is of the same order of magnitude as that of histamine itself. When tested *in vitro* on the isolated guinea-pig gut, one molecule of Pyribenzamine counteracts many moles of histamine. A complete antihistaminic effect can be obtained with doses of .01 micrograms or less per milliliter (of test solution). When introduced into the lungs as an aerosol, a few micrograms of Pyribenzamine will protect for several hours against the lethal effects of histamine aerosol inhalation or intracardially injected histamine. Ten micrograms, injected intra-arterially, stops the histamine-induced salivation in cats. By subcutaneous injection, 3 mg./kg. of Pyribenzamine protect guinea pigs against up to 120 lethal doses of histamine.

The nullification of these histamine effects has therefore been considered as a specific displacement reaction, similar to the action of sulfonamides upon PABA.

*Other Pharmacologic Properties.* Antihistaminic activity is, however, not the only pharmacologic effect produced by this group of compounds. As with most of the other substances with strong pharmacologic action, their pharmacodynamic activities extend to various other physiologic mechanisms. Pyribenzamine has an atropine-like action on smooth muscles and glands, nullifying the spasmodic effects of acetylcholine. It has local anesthetic activity and, furthermore, excites the central nervous system. Although suspected, it has not yet been shown that certain of these so-called "secondary effects" may be intrinsically linked to "antihistaminic" activity.

These secondary pharmacological activities are, however, relatively minor, and the antihistaminic activity represents, in all substances thus far studied, a high peak in the pharmacological spectrum. The anticholinergic power of Pyribenzamine, for instance, as determined *in vitro* on the guinea-pig gut, amounts to only one one-thousandth of its antihistaminic power. This relationship between antihistaminic and antiacetylcholine activity is not constant, and it varies with the different antihistaminic compounds. All degrees and gradations are encountered between substances with very high antihistaminic and low antiacetylcholine activity, and vice versa. In other words, there exist all degrees of transitions from the antihistaminics of the Pyribenzamine type to the antispasmodics of the Trasentine type. Also relatively weak, as compared to the antihistaminic power, is the local analgesic power of Pyribenzamine and the various related antihistaminics. Their potency is one to three times that of procaine, and in this case too, all gradations of antihistaminics with weak local anesthetic power to strong local anesthetics with small antihistaminic power are known.<sup>6</sup>

*Antiallergic Activity.* Substances with such high antihistaminic power should exert strong antianaphylactic activity, provided the histamine theory of anaphylaxis is valid. From the pharmacodynamic analysis of the first antihistaminic substances of this group, 929F. and 1571F, Bovet and Staub<sup>7, 8, 9</sup> had already assumed and seen that they prevented the appearance of all manifestations which are produced by the release of histamine in anaphylaxis.

On the other hand, from purely theoretical speculations, one would expect that these compounds would remain ineffective in all allergies in which allergotoxins other than histamine are liberated and are the cause of allergic manifestations. It should similarly be ineffective in those conditions in which histamine is liberated in relatively large amounts but constitutes only a by-product and remains innocuous, since the animals or the organs in which it is liberated are insensitive to histamine.

As far as allergic manifestations are concerned, laboratory experiments and clinical trials have partially confirmed this hypothesis. Antihistaminic substances have proven to be highly active in all those allergic manifestations which most strikingly resemble histamine effects, that is, anaphylaxis of guinea pigs and anaphylactoid symptoms of man, serum disease, acute urticaria, hay fever, and rhinitis. In addition, however, they proved to be active in other allergic manifestations not believed to be produced by histamine. They were indeed quite effective in certain experimental sensitizations of the epidermal type, namely, contact dermatitis, as well as in certain cases of dermatitis and eczema in humans.<sup>10, 11</sup>

It has been said that, with the use of antihistaminics, we could detect what role histamine plays in various pathological processes. If this were true, the activity of Pyribenzamine in epidermal sensitizations could suggest that, contrary to all that is known thus far, histamine is the allergic poison responsible for many cases of dermatitis, eczema, and other inflammations of the skin where histamine release has indeed been demonstrated but, until now, has been considered a waste product. However, the activity of Pyribenzamine in dermatitis may be attributed to another mechanism. It is possible that this substance prevents or alleviates certain allergic manifestations not only because it counteracts histamine, but also through one of its secondary pharmacologic activities.

An investigation of this alternative possibility resolves itself to the questions as to which physiologic mechanism operates in a special case of allergy and whether the so-called secondary pharmacologic properties of Pyribenzamine, such as the local anesthetic, antispasmodic power, or others, still unknown, play a role in certain therapeutic effects wherein histamine is not involved.

Such a study was carried out with various experimental sensitizations in laboratory animals, representing a much more uniform test subject than human allergy. The following allergies were investigated: (1) anaphylaxis of guinea pigs, as an example of primary sensitization of the smooth muscle; (2) anaphylaxis in mice, as an example of allergy in histamine-resistant animals; (3) urticaria in guinea pigs, as an example of sensitization in which the capillary system of the skin is principally involved; and (4) contact dermatitis in guinea pigs, as an example of an allergy in which the epidermal cells are the primary site of the sensitization.

We shall now discuss these experimental sensitizations in detail.

*Anaphylaxis of Guinea Pigs.* Anaphylaxis of guinea pigs is almost identical with histamine intoxication. Of all forms of experimental allergies, it responds most successfully and promptly to Pyribenzamine in surprisingly

small doses. Complete protection from all symptoms of shock is conferred with one subcutaneous injection of 1 to 2 mg./kg. body weight. In fact, one-tenth of this dosage is capable of suppressing shock in a number of animals.<sup>2, 12</sup> These borderline doses primarily act on the organs which are most sensitive to histamine, namely, the lung. The guinea pigs die of asphyxia long before any of the other histamine or anaphylactic symptoms have had time to manifest themselves.

Since it seems probable that Pyribenzamine protects guinea pigs from anaphylactic death principally because it nullifies histamine liberated in the lungs during anaphylactic shock, it could be expected that the drug would act in much smaller doses if introduced directly into the lungs as an aerosol. This is indeed the case. The inhalation of a 1 to 2 per cent Pyribenzamine aerosol for ten to thirty minutes protects guinea pigs not only against up to 15 lethal doses of histamine intravenously injected,<sup>13</sup> but also against anaphylactic shock when they are actively or passively sensitized to horse serum. The prophylactic effect may last for several hours. In some instances, an aerosol concentration of only 0.1 per cent Pyribenzamine was effective. The amounts of Pyribenzamine absorbed in the lungs have not as yet been measured, but it can be anticipated that they are of the order of micrograms or even fractions of micrograms (FIGURE 1).

These minute amounts introduced into the lungs selectively protect the smooth muscles of the bronchial system but do not protect other organs. Many animals die of delayed anaphylaxis, or, in the case of histamine injections, show signs of systemic histamine intoxication.

The injection of 10 to 15 lethal doses of histamine into guinea pigs protected with Pyribenzamine aerosol produced a deep narcosis which lasted from 30 to 60 minutes. Similarly, sensitized guinea pigs, protected from anaphylaxis by aerosol treatment, have reacted to the challenge with serum by undergoing a long-lasting state of depression, apparently corresponding to the histamine narcosis. It is entirely possible that certain side effects of antihistaminics, especially the drowsiness and dizziness, are related to this hitherto unknown histamine effect.

*Anaphylaxis in Mice.* If it is true that the exceedingly high response of guinea pig anaphylaxis to Pyribenzamine is connected with the high sensitivity of this animal to histamine, then it should be expected that Pyribenzamine would display only little or no antianaphylactic effect in the mouse—an animal resistant to histamine.

Mice tolerate up to 750 mg. per kg. body weight of histamine salt, whereas guinea pigs are killed by one one-thousandth of this dose. Mice, unlike guinea pigs, are extremely resistant to any sensitization, and many authors attribute the great difficulty in inducing allergy in them to their low sensitivity to histamine. Since the total histamine content of the normal mouse is approximately 10 mg. of histamine per kg. of body weight and since mice present the first signs of histamine shock only when 100 or more mg. of free base are injected, it is rather improbable that histamine is involved in mouse anaphylaxis.

We were able to show that Pyribenzamine and Benadryl did not counter-

act histamine intoxication of mice, as is the case with guinea pigs or dogs.<sup>14</sup> On the contrary, they augmented the toxicity of histamine—an observation which recently has been confirmed by Halpern<sup>15, 16</sup> in his toxicity studies with the antihistaminics of the thiodiphenyl series. Anaphylaxis in mice also was essentially unaffected by Pyribenzamine. A slight influence was sometimes observed with near toxic doses, but this effect was not comparable to its high effectiveness in guinea-pig anaphylaxis, where Pyribenzamine was effective in about one four-hundredth of the lethal dose.\*

This result constitutes additional evidence for the assumption that mouse anaphylaxis is caused principally by an anaphylactotoxin other than histamine. It also confirms the finding that Pyribenzamine counteracts ana-

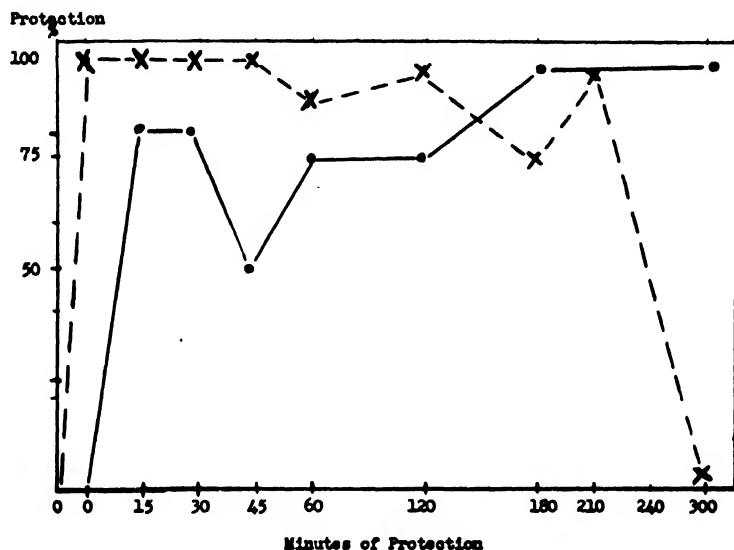


FIGURE 1. Pyribenzamine aerosol. Protection of guinea pigs against histamine intoxication and anaphylactic shock (X = histamine; ● = anaphylaxis). Aerosol: 2 per cent Pyribenzamine by aerosolization as described by Mayer.<sup>2</sup> Inhalation for 30 minutes. Histamine intoxication: 3 mg./kg. histamine phosphate (= 4 lethal doses) intracardially injected at various intervals. Anaphylaxis: Active sensitization to horse serum. Reinjection of 1 ml. of horse serum at various intervals after aerosolization. Abscissa: Time in minutes after the end of aerosol treatment. Ordinate: Percentage of protected inhalation animals. Total number of animals: 173.

phylaxis only when it is induced by histamine. From these experiments, it appears that the activity of antihistaminics in the anaphylactic form of guinea-pig allergy can properly be explained on the basis of a single pharmacologic activity, namely, nullification of histamine effects.

*Urticaria.* Acute urticaria is one of the most responsive clinical indications for antihistaminic therapy. It is assumed, according to all we have learned since the work by Lewis on the urticarial wheal, that the therapeutic effectiveness of antihistaminics in this condition also depends primarily upon the nullification of histamine. Curiously enough, the therapeutic value of the various antihistaminics decreases as the urticaria becomes more chronic, in spite of the fact that the clinical symptoms of acute and chronic urticaria are the same. The question arises whether, in these chronic cases, a shift

has taken place from histamine, as the etiologic factor, to another urticarigenic allergotoxin.

The answer to this problem is still unknown. Thus far it has been possible to produce experimentally only acute urticaria, or at least symptoms very close to acute urticaria. When guinea pigs are sensitized to hog serum and challenged 20 days later with an intradermal injection of the same antigen, an urticarial eruption appears within 16 to 24 hours, which is localized particularly on all shaven parts of the skin, on the ears, and on the visible



FIGURE 2. Influence of Pyribenzamine upon the urticarial manifestation produced in guinea pigs sensitized to hog serum. The animal on the right is a control animal, challenged with hog serum and presenting deep-red ears and mucosae. The animal on the left was treated in the same manner, but received a subcutaneous injection of 15 mg./kg. Pyribenzamine at the height of the allergic manifestation. The skin eruption disappeared within 15 minutes and the skin became almost normal.

TABLE 1

EFFECT IN GUINEA PIGS OF PYRIBENZAMINE UPON THE VASCULAR SKIN REACTIONS AFTER SENSITIZATION WITH HOG SERUM

	<i>Pyribenzamine doses—mg. per kg.</i>					
	<i>0.1</i>	<i>1</i>	<i>5</i>	<i>10</i>	<i>20</i>	<i>25</i>
action	none	none	none in one case, active in another	none in one case, active in three others	active in three cases	active in two cases

15 guinea pigs, sensitized to hog serum and treated, after challenge, with various amounts of Pyribenzamine. Minimal active dose is 5 mg./kg. subcutaneous injection.

mucosae.<sup>17</sup> The histological picture of the lesions is that of a typical urticarial eruption, with primary changes on the capillary system in the form of dilatation of the blood vessels and lymphatics (FIGURE 2). This rash promptly disappears within 10–15 minutes with Pyribenzamine therapy when doses ranging from 5 to 10 mg. per kg. of body weight are injected, and reappears after an interval of several hours unless treatment with the anti-histaminic is repeated.<sup>18</sup> The result of one experiment is given in TABLE 1.

This acute urticaria of guinea pigs is only one manifestation of a general anaphylactic sensitivity produced by the sensitization with hog serum. If

the antigen is injected intracardially and not intradermally into the sensitized animals, they immediately die from anaphylactic shock, and the general anaphylaxis responds to Pyribenzamine as promptly as does the urticaria. This fact, together with the obvious analogy between guinea-pig urticaria and acute human urticaria, makes it quite probable that the therapeutic effect of Pyribenzamine in experimental urticaria results primarily from the nullification of histamine.

*Contact Dermatitis.* The next form of experimental allergy in which the mechanism of action of Pyribenzamine was investigated is experimental contact dermatitis. We have seen that this form of allergy is quite resistant to antihistaminic therapy, and there are certain facts which may explain this resistance. Allergic dermatitis, in many respects, develops differently from either the anaphylactic or urticarial forms. The allergic manifestations of anaphylaxis or urticaria appear explosively within a few minutes after the introduction of the antigen. The allergotoxin—in this case, histamine—is liberated at once and acts immediately. Its effect is functional; the allergic manifestation is the result of an excitation or a paralysis of physiologic mechanisms.

The epidermal allergic challenge reaction becomes visible only after an incubation of many hours and is characterized by inflammatory changes at the site of the reaction. The slow onset of the epidermal reaction is probably due not so much to a slow liberation of the allergotoxin, but rather to the fact that the reactivity of the epidermal cell is very slow. The epidermal allergic manifestation is not due to a functional change, but to destructive cell injuries.

The very first symptoms of allergic or nonallergic dermatitis, namely, the erythema and pruritus accompanying dermatitis and especially atopic eczema, are probably produced by histamine liberated during the irritation or the antigen-antibody reaction. They are promptly taken care of by Pyribenzamine. The nature of the allergotoxin responsible for the specific cell changes in dermatitis and eczema which follow this initial state is unknown, but it is quite probable that it is not histamine. Histamine intoxication is, as we have seen, typified by a series of very acute symptoms such as wheals, increased secretion of certain glands, pruritus, and spasm of various smooth muscles. These delayed symptoms manifested in dermatitis or eczema are not typical histamine symptoms, and no one has yet succeeded in producing dermatitis with histamine, even after prolonged administration. These cellular changes, which constitute the characteristic changes in dermatitis and eczema—spongiosis, intraepidermal vesicles, cell infiltration into the dermis and cutis, *etc.*, are apparently the consequence of the liberation of other allergotoxins.

Experimental sensitizations of the epidermal type can readily be obtained when guinea pigs are pretreated with various chemical substances such as paraphenylenediamine, dinitrochlorobenzene, poison ivy extract, and many other compounds known to produce contact dermatitis in man. This hypersensitivity of the skin develops under conditions identical with those leading to contact dermatitis in human beings. One or more intradermal injections



or application of salve on the eroded skin usually give rise to a generalized hypersensitivity of the entire epidermis.<sup>19, 20</sup>

The manifestations of these experimentally produced types of contact dermatitis in guinea pigs very much resemble those in human beings. After a primary state of erythema and itching, a vesicular dermatitis with infiltration and eosinophilia in the affected tissue develops, which is followed by scaling and full repair.

The influence of Pyribenzamine upon two different kinds of experimental



FIGURE 3. Contact dermatitis-type sensitization of guinea pigs to paraphenylenediamine. Top: animal challenged at the height of the sensitization. Thickening and strong infiltration of the skin with eosinophiles, formation of intraepidermal vesicles. In the inflamed skin, large amounts of oxidative enzymes are present, which produce the rapid blackening of the paraphenylenediamine. Bottom: animal treated in the same manner as above animal, but, simultaneously with the challenge, 2 per cent Pyribenzamine base in olive oil was applied locally. The skin is almost normal; infiltration is only slight and shows much less discoloration.

dermatitis has been studied—one produced by sensitization to paraphenylenediamine (FIGURE 3)<sup>19</sup> and the other by sensitization to 2,4-dinitrochlorobenzene.<sup>21</sup> These two allergies show slight differences in their clinical aspect and in the pathological changes. Paraphenylenediamine dermatitis of guinea pigs is characterized by the regular appearance of intraepidermal vesicles and, thus, very closely resembles dermatitis in man. More persistent erythema is characteristic of dinitrochlorobenzene dermatitis and intraepidermal vesicles are very rare.

In spite of these differences, Pyribenzamine was therapeutically active in both forms. It reduced considerably the intensity of the inflammation and shortened the healing process. It was effective when doses of 15 to 20 mg.

per kg. of body weight were repeatedly given by subcutaneous injection. It was far more effective, however, when applied locally at the site of the reaction. Solutions of Pyribenzamine base in oil or Pyribenzamine ointment were capable of preventing in a number of animals any signs of dermatitis; in others, they considerably reduced the inflammation. The protocol of one experiment is indicated in TABLE 2. As a consequence of these experiments, the use of Pyribenzamine ointment was proposed for human contact dermatitis and eczema. Lately, Feinberg and Bernstein<sup>22</sup> have reported good results with the topical use of Pyribenzamine.

How can we explain the activity of antihistaminic substances in eczema or contact dermatitis? Shall we suppose, contrary to laboratory evidence, that histamine is instrumental in the production of dermatitis and eczema, as it is in anaphylaxis and urticaria? And shall we believe that high doses of Pyribenzamine and prolonged use are required in dermatitis because con-

TABLE 2  
INFLUENCE OF PYRIBENZAMINE UPON EXPERIMENTAL CONTACT DERMATITIS AFTER SENSITIZATION OF GUINEA PIGS WITH P-PHENYLENEDIAMINE. PROTECTION AFTER LOCAL TREATMENT WITH 2 PER CENT PYRIBENZAMINE IN OIL

Number of animals	Pretreatment	Pyribenzamine administered	Animals showing:							
			Dermatitis				Diamine oxidation			
			++++*	++	+	0	++++	++	0	(+)
2	None (Not sensitized)	None				2				2
5	Sensitized	None	5				5			
5	Sensitized	2% Pbz. in oil		1	4			1	4	

\* + to ++++ represent different degrees of reaction to paraphenylenediamine.

siderable cell repair is necessary to restore normality? In an earlier study, we accepted these explanations, but today, as a result of recent studies, we are skeptical. It now seems more probable that Pyribenzamine acts in epidermal sensitizations by virtue of pharmacologic properties different from its antihistaminic power.

As mentioned in the introduction, the antihistaminic substances are endowed with a great array of pharmacologic properties, in addition to their most striking property of counteracting histamine. Certain of these secondary activities are by no means unimportant attributes for antiallergic agents. There is, for instance, the matter of antiacetylcholine activity. Various theories have been advanced claiming that acetylcholine, rather than histamine, is the ultimate cause of anaphylactic shock or of certain forms of urticaria.

It is not our intention to discuss this theory now, but there are many facts which make it improbable that the antiacetylcholine activity of Pyribenzamine and other antihistaminics plays an important role in any of their therapeutic antiallergic effects. Atropine, Trasentine, and other antispasmodics

have no, or at least very little, effect in anaphylaxis and dermatitis. Furthermore, antihistaminic substances with relatively high antiacetylcholine activity, as, for instance, Benadryl, are not stronger antiallergics than substances with low antiacetylcholine activity such as Pyribenzamine. In so far as the role of acetylcholine as a dermatitic agent is concerned, it has not been shown to be more instrumental in producing dermatitis than histamine.

It is more difficult to answer the question as to what extent the local anesthetic properties of antihistaminics may account for their antidermatitic activities. Local anesthetics are known to prevent or diminish anaphylactic shock and dampen other allergic reactions in animals. Similarly, they favorably influence certain forms of allergic eczema, especially the so-called "lichen Vidal" forms of atopic eczema. As we have seen, all antihistaminics within the structural family of Pyribenzamine are local anesthetics.

The therapeutic effect of Pyribenzamine aerosols in anaphylaxis is not due to a local anesthetic component, since procaine aerosols were entirely without effect. In dermatitis, the question is not so simple. The local anesthetic activity of Pyribenzamine may well play a certain role when it is applied locally in the form of an ointment, but it is more than questionable whether this activity is implicated when Pyribenzamine is administered parenterally, since no anesthetic or analgesic effect can be observed under these conditions.

It is probable that other, still unknown pharmacodynamic effects of Pyribenzamine are operating in its antidermatitic activity. In investigating this question, two facts may be borne in mind: (1) dermatitis and eczema are general and unspecific inflammatory processes which are not confined to an allergic process; and (2) besides histamine, several other toxic products have been isolated from tissue during inflammation which, unlike histamine, are capable of producing inflammation in normal tissue.<sup>23, 24</sup>

The clinical signs of an allergic dermatitis and eczema are not different from those present in primary, nonallergic irritations of the skin. Since the same pathologic process can result from various and entirely different processes, allergic and nonallergic, it may be assumed that the substances directly responsible for this form of skin inflammation are liberated not only during a primary nonallergic irritation of the skin, but also during an allergic skin irritation following an antigen-antibody reaction.

Various substances have been isolated from primary inflammatory tissue, such as leucotaxine, leucotropine, necrosin, and proteolytic enzymes. A study investigating whether these same substances may also be released and be operative in allergic dermatitis is now in progress. So far, only negative results have been obtained in experiments on the influence of antihistaminic substances upon some of these products connected with inflammation. Culumbine<sup>25</sup> has investigated the influence of Neontanergan upon leucotaxine, and Loew,<sup>26</sup> that of various antihistaminics upon the skin reaction to trypsin. No effect was observed in either case.

Another enzyme which is released and plays an active role in certain inflammations of skin and other tissues is hyaluronidase. It has been known for a long time that the skin presents a natural barrier to bacterial invasion and subsequent inflammation. The principal component of this barrier is hyaluronic acid, which is present in connective fibers and mucoid tissue. In

order to overcome this barrier, invasive bacteria produce or liberate an enzyme, hyaluronidase, which hydrolyzes hyaluronic acid, thus weakening the barrier and permitting invasion of the tissue or spread and diffusion of toxins.<sup>27, 28</sup> Hyaluronidase, however, is not only a product of certain bacteria, but is contained, in bound form, in many cells of the animal organism, from which it is released under certain physiologic conditions. Skin, for instance, is very rich in hyaluronidase.

Various phenomena of spreading are well known in allergic and nonallergic inflammations, especially in dermatitis and eczema. The question therefore naturally arises whether hyaluronidase may be liberated by some nonliving agents and thus play a role in nonbacterial inflammations.

We have studied this problem on guinea pigs sensitized to paraphenylenediamine and challenged later with an intradermal injection of the antigen.

TABLE 3  
INFLUENCE OF HYALURONIDASE ON THE CHALLENGE REACTION IN EXPERIMENTAL  
EPIDERMAL SENSITIZATION TO PARAPHENYLENEDIAMINE  
(PP) IN GUINEA PIGS

	Num- ber of ani- mals	Average size and intensity of local reaction			
		Without hyaluronidase		With hyaluronidase	
		Size in cm <sup>2</sup>	Inten- sity of inflam- mation	Size in cm <sup>2</sup>	Intensity of inflammation
Controls.....	4	0.317 ± 0.12	(+)	1.60 ± 0.14	+
Sensitized animals.....	18	2.3 ± 0.74	+	16.9 ± 0.52	+++++

Challenge: 21 days after sensitization, 0.7 mg. of PP in 0.15 ml. of fluid.

Hyaluronidase: 0.7 mg. per 0.15 ml. of liquid injected.

Figures = cm<sup>2</sup> × 10.

(+)-++++ represent different degrees of inflammation (redness + infiltration).

In the presence of hyaluronidase, the size of the allergic, inflammatory reaction was more than 7 times greater than in the absence of this enzyme, which proves that hyaluronidase increases not only the spread of India ink, but also the spread of the antigen, paraphenylenediamine. Of special interest was the further observation that, in spite of the dilution of the antigen in the tissue and the seven-fold increase in reactive area, the inflammatory reaction was not decreased; on the contrary, it was increased several-fold. In the 18 animals tested, the infiltration and the redness were so much more intense that the allergic reaction increased from + to a strength of +++ or ++++ (TABLE 3).

Although it is not possible to draw definite conclusions from these experiments without a study of the comparative hyaluronidase content in normal and inflamed tissue, the results presented are an interesting indication of the possibility that hyaluronidase is involved in this allergic inflammation.

Since this experiment, we have investigated the influence of Pyribenzamine and Antistine upon the action of hyaluronidase<sup>28</sup> and have found that both

antihistaminics inhibited the action of the enzyme, upon the spreading of India ink in the rat's skin, by almost 50 per cent—an action similar to that of morphine<sup>30</sup> and salicylic acid.<sup>31</sup> FIGURES 4 and 5 illustrate the results of an experiment with Pyribenzamine, and TABLE 4 shows the decrease of the India-ink spread under the influence of Pyribenzamine.



FIGURE 4. Rat: right side, before Pyribenzamine; tail—India ink plus saline; head—India ink plus 1 per cent hyaluronidase. (Dark spot upper center is due to the subsequent injection of Pyribenzamine.)



FIGURE 5. Rat: left side, after 37.5 mg. of Pyribenzamine; tail—India ink plus saline; head—India ink plus 1 per cent hyaluronidase. (This photograph has purposely been reversed in order to permit a comparison of FIGURES 4 and 5.)

The action of Pyribenzamine upon the hyaluronidase spread of the allergic skin reaction was still more important. This reaction was decreased to almost one-sixth of the controls. In an experiment with 18 sensitized guinea pigs, the reaction size decreased from 17 cm.<sup>2</sup> and three-plus to almost 3 cm.<sup>2</sup> and less than one-plus. TABLE 5 shows the results of this experiment and the comparison with the reaction before Pyribenzamine. This result is in-

teresting and important, since, according to Duran-Reynals,<sup>27</sup> it seems reasonably certain that the spreading effect of hyaluronidase is not connected with histamine. The amounts of Pyribenzamine capable of preventing the effects of hyaluronidase upon the spread of India ink and upon the size and intensity of the allergic skin reactions in animals sensitized to paraphenylene-

TABLE 4

EFFECT OF PYRIBENZAMINE, ANTISTINE, AND MORPHINE UPON THE SPREADING OF INDIA INK IN THE PRESENCE AND ABSENCE OF HYALURONIDASE

Dosage mg./kg.	No. of animals tested*	Per cent reduction of spreading area	
		Spreading of India ink alone	spreading of India ink plus hyaluronidase
Pyribenzamine			
75	20	47.85 $\pm$ 2.53	43.95 $\pm$ 3.73
37.5	20	18.70 $\pm$ 5.50	21.10 $\pm$ 3.77
15	20	14.05 $\pm$ 4.21	6.25 $\pm$ 5.39
Antistine			
75	20	28.10 $\pm$ 4.99	37.00 $\pm$ 3.22
37.5	20	31.75 $\pm$ 5.22	20.00 $\pm$ 4.23
15	20	13.65 $\pm$ 5.73	11.10 $\pm$ 4.77

\* Experiments in rats. Each figure represents the mean of the results obtained with 20 animals.

TABLE 5

INFLUENCE OF PYRIBENZAMINE UPON THE ALLERGIC PARAPHENYLENEDIAMINE (PP) REACTION IN PRESENCE AND ABSENCE OF HYALURONIDASE IN GUINEA PIGS

	Number of animals	Average size and intensity of local reaction			
		without hyaluronidase		with hyaluronidase	
		size in cm <sup>2</sup>	inten- sity of inflam- mation	size in cm <sup>2</sup>	intensity of inflammation
Controls.....	4	0 (0.317)	0 ((+))	0.75 (1.4)	0 (+)
Sensitized animals.....	18	0.27 (2.3)	0 (+)	2.7 (16.9)	+ (+ + + + + + +)

Pyribenzamine: 15 min. before and 4½ hours after challenge, 15 mg./kg. subcutaneously.

Figures =  $\pi r^2 \times 10$ .

Figures and signs in ( ): results before Pyribenzamine.

The control values represent the mean obtained with 4 animals; those for the sensitized animals represent the mean of 18 animals.

diamine were the same as those which influenced the epidermal contact dermatitis in guinea pigs.

Thus, these experiments have shown that Pyribenzamine and Antistine possess, in addition to their antihistaminic activity, at least one other property which may explain their action in various manifestations not as yet associated with histamine release. The strong anti-hyaluronidase effect,

which results in a reduction of the size as well as of the intensity of an allergic inflammation, may well explain their activity in epidermal sensitizations such as eczema or contact dermatitis.

These are the results of the various experiments on the influence of antihistaminics upon experimental sensitizations. Many problems remain unsolved. Nevertheless, we may attempt to answer the question which we raised earlier in our discussion, namely: how might the pharmacologic spectrum of antihistaminics account for their antiallergic activity?

If we recall at this point the various pharmacodynamic properties of the different antiallergic compounds, there is, in our opinion, little doubt that Pyribenzamine acts in anaphylaxis, urticaria, pruritus, and the very first stages of dermatitis by nullification of histamine effects. This action against histamine is the principal pharmacologic and antiallergic property of Pyribenzamine and therefore is the principal basis of its specificity as an antiallergic agent. Other pharmacologic actions, however, are probably responsible for the activity of Pyribenzamine in certain forms of allergic manifestations not connected with histamine.

Dermatitis and eczema are allergies in which, apparently, not histamine, but other allergotoxins are liberated and are responsible for the manifestations. Hyaluronidase appears to be one of these dermatitic allergotoxins. If this be the case, then the antihyaluronidase and anti-inflammatory effect of Pyribenzamine and Antistine may partially or perhaps fully explain their antidermatitic or antieczematic activity.

The mass of literature on the mechanisms of action of antihistaminic substances is growing rapidly. Many facts have been accumulated concerning their pharmacology and antiallergic nature. We are now entering the phase where we find it necessary to deepen and at the same time expand our conception of their mode of action in allergies. As histamine is not the complete answer for allergy, neither does the antihistaminic power entirely explain the antiallergic activity of the so-called "antihistaminic substances." Very few drugs act by a single mechanism. As a rule, the clinical relief which is obtained upon their use is the result of the accumulation of various activities and of simultaneous and concerted attacks upon different cell and enzyme systems. Antihistaminic substances are no exception to this rule, and we believe that they are antiallergic remedies because they attack the allergic process from different directions. There is no doubt in my mind that, in spite of this fact, histamine is the principle point of attack of antihistaminics.

Allergy is one of the most complicated and most varied forms known of all the pathologic processes. It would be too simple to reduce this great complexity to the single formula, histamine. The same consideration applies to antihistaminic substances. At first, the problem was oversimplified and all therapeutic effects were explained on the basis of antihistaminic activity. The more we learn about these substances, however, the more we realize that their antiallergic action appears as complicated and as varied as allergy itself.

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# THE PHARMACOLOGY OF BENADRYL AND THE SPECIFICITY OF ANTIHISTAMINE DRUGS

By Earl R. Loew\*

*Department of Pharmacology, University of Illinois, College of Medicine, Chicago, Illinois*

Designation of compounds as antihistamine drugs, antihistaminics, or histamine antagonists implies that such drugs exert a specific inhibiting influence on the actions of histamine. The term, specificity, as herein used, implies that a drug may exert a definite type of effect, such as antihistamine action, and to a degree which is in sharp contrast to one or more effects which are less conspicuous, not necessarily related and possibly not of pronounced biological significance. The degree of specificity is frequently ascertained (1) by comparing the magnitude of the specific effect of the drug with the lesser effects, all effects being induced by comparable doses of the drug; or (2) by determining the amount of drug necessary for eliciting each effect and noting the relative differences between such amounts, in which case the quantity of drug required to elicit the specific effect should be only a small fraction of that needed to elicit the lesser effects.

Degree of drug specificity is important from several standpoints. A drug which exerts a single type of action can frequently be used effectively as a research tool to produce or prevent a physiological or pharmacological response, the analysis of which the investigator is desirous of undertaking. When specific, proper doses of a drug will evoke the desired response without eliciting other effects to a degree which may render interpretation difficult or impossible. Drugs with a high degree of specificity frequently prove useful as diagnostic agents, since they may enhance or diminish existing dysfunction and their single type of action renders interpretation more reliable. Some valuable therapeutic agents are nonspecific. Nevertheless, from a therapeutic standpoint, a specific drug may prove to be especially useful by eliciting desired effects without adding the complicating feature of untoward reactions which are related to several actions of a drug.† The specific effect of antihistamine drugs, as well as their less conspicuous actions, are important from the viewpoint that thorough analysis may provide important information concerning their mode of action.

In substance, the specificity of antihistamine drugs is of importance in relation to their mode of action, their use as research tools, and their application as diagnostic and therapeutic agents. A single example will serve to emphasize the significance of antihistamine specificity. If a reasonable degree of specificity has been demonstrated for antihistamine drugs, their effec-

\* It is not the intention to imply that specific drugs fail to induce undesired side-effects, since it is common knowledge that a drug as specific as atropine, when used as an antispasmodic, may induce tachycardia, mydriasis, and xerostomia. Such undesired responses are due to the antiacetylcholine action of atropine, a drug which is specific but which lacks selectivity for a given tissue and therefore alters activity in a number of tissues, each of which depends upon the action of acetylcholine for normal activity. By analogy, there is the possibility that a specific antihistamine drug would also induce diverse responses in the animal organism, but this is largely dependent on whether histamine is intimately related to the physiological economy of the animal organism.

† Present address: Department of Physiology, Boston University School of Medicine, Boston, Massachusetts.

tiveness in controlling manifestations of experimental anaphylaxis and clinical allergy would constitute definite support for the belief that histamine is an important factor involved in such conditions.

The realization that antihistamine drugs might exert several types of action to some degree stemmed from considerations of chemical structure and pharmacological actions actually demonstrated. The chemical nature of most antihistamine compounds immediately suggests the possibility that atropine-like, antispasmodic, local anesthetic, or sympathomimetic actions might be demonstrable. One of the early reports from Dr. Bovet's laboratory by Staub<sup>56</sup> mentions that the phenolic ethers and ethylenediamine derivatives were systematically examined for sympathomimetic and sympatholytic action. This is an important point, since developments made during the past decade indicate that all antihistamine drugs either enhance or diminish some of the excitatory effects of epinephrine.

The original antihistamine drugs, 929F, 1571F, and Antergan actually exhibited several pharmacological properties other than the antagonism of histamine.<sup>20, 55</sup> Confirmation and extension of the experimental findings with these drugs, as well as evaluation of the antihistamine drugs developed more recently (for refs. cf. Loew<sup>38</sup>), reveal the necessity of considering several pharmacological properties in addition to antagonism of histamine. A recent review of the literature<sup>38</sup> reveals that antihistaminics can produce several pharmacological effects. For purposes of orientation and development of the discussion concerning the specificity of antihistamine drugs, the pharmacological properties which appear to deserve consideration in relation to antihistamine action are herein outlined:

(1) An atropine-like action is indicated by the ability of a drug to prevent acetylcholine from exerting its usual secretory, depressor, mydriatic, or spasmogenic action. Only a very low, and probably insignificant, degree of anti-acetylcholine action is revealed in the literature dealing with 929F,<sup>49, 50, 55</sup> 1571F,<sup>20, 38, 55, 58</sup> Antergan,<sup>20</sup> Antistine,<sup>4, 9, 44</sup> Neoantergan,<sup>8, 13, 52, 53</sup> and Pyribenzamine.<sup>41, 53, 61</sup> The same is true of the thiophene analogue of Pyribenzamine studied by several groups of investigators,<sup>17, 29, 32, 51</sup> as well as the halogenated thiophene analogues examined by Litchfield and co-workers.<sup>32</sup> The weak, atropine-like action of Benadryl is less than one-fiftieth or one one-hundredth of that exhibited by atropine itself. Evidence of such atropine-like action of Benadryl includes the antagonism of the spasmogenic effects of acetylcholine<sup>10, 38, 58</sup> and the antagonism of its depressor action.<sup>38, 58</sup> In addition, mydriasis has been demonstrated in rabbits<sup>35</sup> and man,<sup>24, 43</sup> and Benadryl inhibited salivary secretion induced by cholinergic influence in animals<sup>8, 14</sup> and occasionally caused xerostoma in patients. The evidence now available is insufficient to warrant any conclusion concerning the degree of atropine-like action exerted by derivatives of thiodiphenylamine (phenothiazine), described by Halpern,<sup>21-23</sup> or by the compound, Thephorin, developed by Lehmann and associates.<sup>30</sup>

(2) The antispasmodic action of antihistamine drugs appears to be related to the degree of atropine-like action which has just been considered

briefly. It is now quite apparent that general antispasmodic or spasmolytic action is not proportional to the degree of antihistamine action, since potent antihistamine drugs may exert only a weak antispasmodic action or actually induce spasm of smooth muscle.

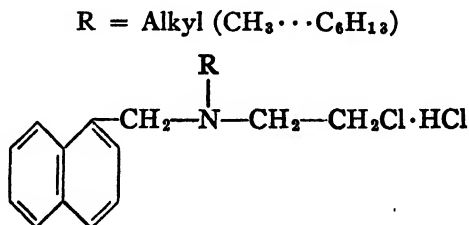
(3) A spasmogenic action is demonstrable when several ethylenediamine derivatives are added to fluids used to bathe or perfuse smooth muscle of isolated lungs, intestines, or uterus.<sup>18, 20, 29, 41, 55, 62</sup> Dilutions which induced spasm usually ranged from 1:20,000 to 1:100,000. Higher dilutions failed to induce spasm and were capable of blocking the spasmogenic effects of histamine. In intact animals, spasm of intestines and the uterus has also been observed.<sup>58</sup> The fact that several potent antihistamine drugs have the propensity of exerting a spasmogenic action is an added reason for not classifying antihistamine drugs as general antispasmodics.

(4) Local anesthetic action is exerted to some degree by all antihistamine drugs, but, unfortunately, very few quantitative studies concerning potency and duration of action have been made. When Benadryl was injected intradermally in man, Leavitt and Code<sup>27, 28</sup> found its local anesthetic action to be at least twice as strong as that exerted by Procaine. The local anesthetic activity of Neoantergan was three times that of Procaine as determined by the intradermal test in guinea pigs.<sup>18</sup>

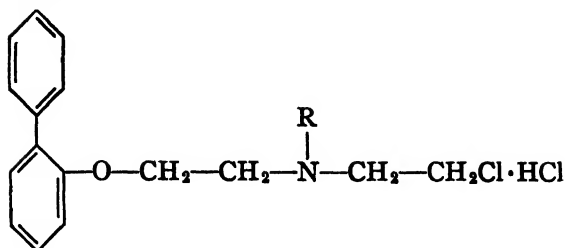
(5) An enhanced and prolonged pressor response to epinephrine occurs (see Loew<sup>53</sup> for refs.) in anesthetized dogs and cats treated with Antergan, Neoantergan, Benadryl, Antistine, Pyribenzamine, and the thiophene analogue of the latter drug.<sup>29</sup> The cause of this phenomenon has not been ascertained, but evidence has been adduced to indicate that the effect is probably unrelated to any atropine-like action.<sup>58</sup> Furthermore, some antihistamine drugs do not elicit this effect.

(6) Diminution of the pressor responses to epinephrine occurs in dogs treated with 929F and Thephorin.<sup>7, 30</sup> This effect can be considered in conjunction with the fact that compounds have recently been discovered which not only antagonize histamine but also block the pressor action of epinephrine and even reverse the pressor to a depressor response.

(7) Sympatholytic or adrenergic blocking action was found to be a prominent effect of certain N-alkyl derivatives of  $\alpha$ -naphthylmethyl- $\beta$ -chloroethylamine and  $\beta$ -(2-biphenyloxy)ethyl- $\beta$ -chloroethylamine.<sup>2, 33, 39</sup> The structural formulae of these compounds are as follows:



$\alpha$ -naphthylmethyl-alkyl- $\beta$ -chloroethylamine  $\cdot$  HCl



$\beta$ -(2-biphenyloxy)ethyl-alkyl- $\beta$ -chloroethylamine  $\cdot$  HCl

These compounds, especially those in the naphthyl series, were also highly effective in diminishing certain effects of histamine. It is possible, however, that they cannot be properly designated as antihistamine drugs, since the adrenergic blocking action was so conspicuous. The ability of these compounds to prevent several effects of histamine and the excitatory effects of epinephrine renders them useful as research tools. For instance, it is inconceivable that the ability of these  $\beta$ -chloroethylamines to antagonize histamine is related to any enhancement of the pressor effects of epinephrine, since these compounds actually reverse the pressor effects of epinephrine.

Admittedly, the drugs which antagonize histamine may elicit one or more of a variety of effects. The degree of specificity against histamine will therefore be dependent on the prominence of antihistamine action in relation to other effects. The most important effects of histamine which have been demonstrated to be diminished or annulled by antihistamine drugs<sup>33</sup> are as follows: (1) spasm of smooth muscle located in the bronchioles, small intestine, and uterus; (2) relaxation of arteriolar smooth muscle, as indicated by a depressor response to histamine or increased vascularity of a region; (3) increased permeability of capillaries, as indicated by wheal formation and transudation of dyes from the vascular system into localized cutaneous areas into which histamine has been introduced; (4) Dilatation of capillaries and other vessels, as evidenced by localized flare reactions and extensive erythematous responses over cutaneous areas.

Antihistamine drugs do not diminish or abolish the effects of histamine on all types of effector cells, since they fail to diminish histamine-induced gastric secretion to a significant degree; and there is not adequate evidence to prove that they diminish the secretagogue action of histamine on pancreatic, lacrimal, and salivary secretory cells.<sup>16, 33</sup>

The diversity of effects of histamine which can be diminished or abolished by antihistamine drugs at once suggests that these agents possess some specific property. It will prove of value to examine detailed evidence concerning the relative specificity of antihistamine drugs in relation to the various effects of histamine.

#### A. Smooth Muscle Spasm Induced with Histamine

(1) *Bronchospasm.* Usually, antihistamine drugs have been selected and partially evaluated by determining their effectiveness in alleviating

severe symptoms or preventing death due to bronchioconstriction, which is so readily induced in guinea pigs with histamine, either administered or liberated during anaphylaxis.

In the experiments designed and executed during the study of Benadryl and related benzhydryl alkamine ethers, various drugs were included in order to assist in determining whether diminution of histamine-induced bronchioconstriction could be accounted for by an antispasmodic, atropine-like, or local anesthetic action. The data in TABLE 1 permit a comparison of the minimal doses of drugs which reduce mortality in guinea pigs subjected to an aerosol of histamine.

It is significant to note that the minimal doses of Neoantergan and Bena-

TABLE 1  
MINIMAL DOSES OF VARIOUS DRUGS WHICH REDUCE SEVERITY OF HISTAMINE SHOCK IN GUINEA PIGS

<i>Drug</i>	<i>Route*</i>	<i>M.E.D.</i> (mg./kg.)	<i>Remarks</i>
Benadryl·HCl	i.p.† s.c.	1.5 0.5	effective at low dose
Neoantergan·H <sub>2</sub> PO <sub>4</sub>	i.p.† s.c.	0.075 0.025	effective at low dose
Atropine·SO <sub>4</sub>	i.p.†	15.0	low effectiveness
Trasentin·HCl	i.p.†	>50.0	noneffective
Procaine·HCl	i.p.†	>25.0	noneffective
Epinephrine·HCl	i.p.†	0.1	partial protection with toxic dose
α-Naphthylmethylethyl-β-chloroethylamine·HCl s.c.		0.025	pronounced antagonism of histamine with a potent adrenergic blocking drug

\* Intraperitoneal and subcutaneous doses administered 15 and 30 minutes, respectively, before exposure to histamine aerosol.

† Data from *J. Pharmacol. & Exper. Therap.* 86: 1, 1946 and 89: 247, 1947.

dryl which protected guinea pigs against histamine are comparatively small (0.025 to 1.5 mg./kg.). Under these experimental conditions, Pyribenzamine<sup>33</sup> and its thiophene analogue<sup>29</sup> were more effective than Benadryl, whereas Antergan exhibited about equal effectiveness.<sup>34</sup> In short, all the recently developed antihistamine drugs were effective in doses less than 1.5 mg./kg.

This protective action cannot be due to an antiacetylcholine action of the antihistamine drugs. First, a comparatively large dose of atropine (15.0 mg./kg.), an exceedingly potent antiacetylcholine agent, conferred only a low protective action in the animals (TABLE 1). This might be ascribed to the antispasmodic action and the direct antagonism of histamine, which has been demonstrated frequently with large amounts of atropine. Secondly, when tested on isolated intestinal muscle, the antispasmodic, Trasentine, exerted more antiacetylcholine action than Benadryl,<sup>35, 36</sup> yet a large

dose of Trasentine failed to prevent or relieve histamine-induced bronchioconstriction.<sup>36</sup> Thirdly, Castillo and de Beer<sup>10</sup> have demonstrated that Benadryl in a dilution of 1:15,000,000 exerted antihistamine action when tested on smooth muscle of tracheal rings, which allegedly reacted in a manner similar to smooth muscle of bronchi. However, sixty times as much Benadryl was required to prevent the spasm induced with acetylcholine, and even larger amounts of Benadryl failed to exhibit an antispasmodic action by relaxing the undrugged tracheal muscle. These facts permit the conclusion that the ability of Benadryl to reduce histamine-induced spasm of bronchioles and bronchi is not due to an antiacetylcholine action. Furthermore, Benadryl probably exerts more antiacetylcholine action than several other antihistamine drugs,<sup>18, 20, 41</sup> so it is obvious that the arguments just stated strongly support the belief that a specific antihistamine action explains the protective action of 1571F, Antergan, Neoantergan, Benadryl, and Pyribenzamine. Halpern<sup>20</sup> also demonstrated that Antergan was highly effective in relieving bronchioconstriction due to histamine, whereas it was of low effectiveness in reducing bronchioconstriction induced with acetylcholine in eserized guinea pigs.

The local anesthetic action of antihistamine drugs, even if especially prominent, could scarcely account for the diminution of histamine-induced bronchioconstriction, since cocaine, Nupercaine, and Procaine (TABLE 1) all failed to prevent the bronchioconstriction in intact guinea pigs.<sup>36</sup> The antispasmodic, Trasentine, also exerts a strong local anesthetic action,<sup>19</sup> but it failed to prevent bronchioconstriction.

Sympathomimetic drugs such as ephedrine and epinephrine cannot be classed as antihistamine drugs, since their ability to diminish various effects of histamine is due to their strong bronchiodilator and vasoconstrictor actions and their ability to decrease capillary permeability. These actions directly oppose those of histamine, which constitutes a physiological antagonism which has not been demonstrated with antihistamine drugs.

Although epinephrine is an exceedingly potent bronchiodilator agent, it failed to relieve histamine-induced bronchioconstriction except with doses so large as to induce symptoms of epinephrine intoxication. No dose of epinephrine was found which would prevent death in all guinea pigs treated with histamine, whereas well-tolerated doses of several antihistamine drugs conferred full protection. Halpern<sup>22, 23</sup> has recently pointed out that antihistamine drugs were more effective than epinephrine and Aleudrine in preventing histamine intoxication in guinea pigs. The evidence indicates that antihistamine drugs specifically antagonize histamine, whereas the conspicuous but nonspecific bronchodilator and bronchiodilator action of epinephrine accounts for antagonism of barium, acetylcholine, and histamine. The antispasmodic or bronchiodilator action of Benadryl must be negligible, since it was just perceptible when large doses (10 mg.) were infused through isolated guinea-pig lungs,<sup>18</sup> no antispasmodic action was demonstrated on tracheal rings,<sup>10</sup> and Benadryl failed to increase the vital capacity in asthmatic patients.<sup>31</sup>

There is no convincing evidence that certain antihistamine drugs exert

a sympathomimetic action. Ethylenediamine derivatives and Benadryl increase the magnitude and duration of the pressor response to epinephrine in anesthetized dogs (see Loew<sup>33</sup> for refs.). The cause of this response remains unexplained. Even if it is proven that the phenomenon is an expression of a weak sympathomimetic action, it would not account for the strong antihistamine action of Benadryl and ethylenediamine derivatives. We have demonstrated<sup>2</sup> that  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine was exceedingly effective in alleviating both histamine shock (TABLE 1) and anaphylactic shock, even though this compound not only failed to exert sympathomimetic action but suitable doses in dogs actually blocked the excitatory effects which follow injections of epinephrine and stimulation of adrenergic nerves.

Lehmann *et al.*<sup>30</sup> have reported that Thephorin decreased the severity of histamine-induced bronchioconstriction. No significant degree of sympathomimetic action has yet been demonstrated with Thephorin, whereas it did diminish the pressor response to epinephrine in cats; a fact which the author has confirmed in dogs.<sup>34</sup> The pressor effects of epinephrine in dogs was also diminished by 929F,<sup>7, 11</sup> but the effect may have been nonspecific and related to hypotension.

Thus, an antagonism of histamine has been demonstrated with 929F,  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine, and Thephorin. Thephorin and 929F diminished the pressor action of epinephrine, and the naphthyl derivative even reversed such pressor action and blocked or reversed effects of adrenergic nerve stimulation. On the other hand, a number of antihistamine drugs enhance the pressor responses to epinephrine. Thus, antihistamine action is probably independent of either a sympathomimetic or adrenergic blocking action.

In summary, it becomes apparent from the pharmacological data relating to responses of bronchiolar smooth muscle that various drugs exhibit a demonstrable degree of antihistamine specificity, since the antagonism of histamine cannot be accounted for by atropine-like, antispasmodic, local anesthetic, sympathomimetic, or adrenergic blocking (sympatholytic) action. Convincing support for the thesis that antihistamine drugs are relatively specific is the fact that no pharmacological action other than histamine antagonism has been demonstrated in intact guinea pigs with the small doses of drugs required to prevent the bronchioconstrictive action of histamine.

(2) *Intestinal Spasm.* Additional information concerning the relative specificity of antihistamine drugs has been secured by determining their ability to diminish intestinal spasm induced with histamine, acetylcholine, and barium. The early studies with 929F and 1571F revealed that these drugs diminished the spasmogenic effects of histamine, and, from reports by Staub,<sup>55</sup> Halpern,<sup>20</sup> and Climenko *et al.*,<sup>11</sup> it is apparent that they are not absolutely specific, since suitable quantities of the drugs also antagonize the spasmogenic action of acetylcholine and barium. The degree of specificity can be expressed as the ratio of the amount of drug required to antagonize acetylcholine or barium to that required to antagonize histamine.

Thus, Halpern<sup>20</sup> demonstrated that it required more than one hundred times as much Antergan to antagonize barium and acetylcholine as was needed to antagonize histamine. Such relationships can be derived from the data obtained by the author and co-workers<sup>28, 58</sup> when comparisons were made of the effects of Benadryl, 1571F, and several antispasmodics on isolated guinea-pig ileum. FIGURE 1 illustrates the relative degree of effectiveness of Benadryl, 1571F, and atropine in diminishing contractions induced by adding histamine, acetylcholine, or barium to the isolated guinea-pig ileum. Benadryl exhibited an appreciable degree of antihistamine specificity, as indicated by the fact that it required 12.5 and 400 times as much Benadryl to antagonize acetylcholine and barium, respectively, as was needed to antagonize histamine. Results with 1571F indicated greater antihistamine

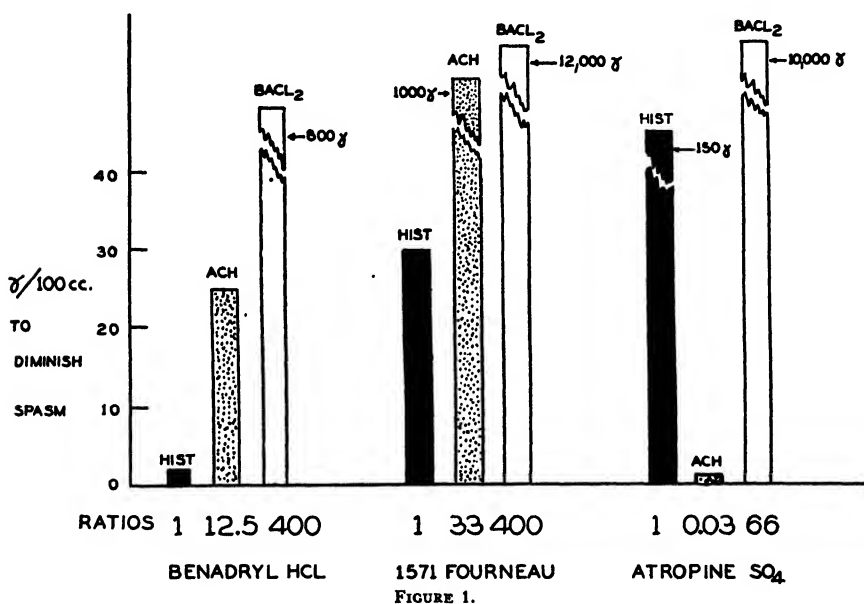


FIGURE 1.

specificity, since it required 33 times as much 1571F to antagonize acetylcholine and 400 times as much 1571F to antagonize barium as was needed to antagonize histamine. Thus, Benadryl exhibited greater antihistamine potency but less antihistamine specificity than 1571F.

Atropine has long been recognized as a potent and highly specific anti-acetylcholine drug. Minute quantities antagonize acetylcholine but the specificity is not absolute, as indicated by the fact that atropine antagonizes histamine and barium when the amounts of atropine are increased 300 and 20,000 times, respectively (FIGURE 1).

It can be concluded from the effects of 1571F and Benadryl on isolated intestinal muscle that a moderate degree of antihistamine specificity has been demonstrated. A similar degree of antihistamine specificity has been demonstrated for Benadryl by Castillo and de Beer,<sup>10</sup> who made extensive



comparisons of drug antagonism on tracheal muscle of the guinea pig. With intestinal muscle, the degree of antiacetylcholine action exerted by Benadryl was much weaker than that exerted by gastrointestinal antispasmodics such as Pavatrine and Trasentine.<sup>88</sup> However, it probably would exceed that exerted by Neoantergan and Pyribenzamine. The information available indicates that Pyribenzamine and Neoantergan only weakly antagonize the spasmogenic action of acetylcholine, and, in view of the strong antihistamine action demonstrated,<sup>8, 41, 42, 53, 59</sup> it is highly probable that quantitative studies would readily provide convincing evidence of a relatively high degree of antihistamine specificity. A high degree of potency and specificity has been demonstrated with Neoantergan.<sup>52</sup>

Attention is drawn to a paper by Schild,<sup>52</sup> which describes methods for determining and expressing both the potency and specificity of antagonistic drugs. Experiments with guinea-pig ileum revealed that Benadryl was 50 times as active against histamine as it was against acetylcholine. Of particular interest is the demonstration that Neoantergan was more potent and more specific against histamine than atropine was against acetylcholine.

The evidence available to date does not indicate that any of the antihistamine drugs has a pronounced ability to diminish the spontaneous motility and tonus of smooth muscle either *in vitro* or *in vivo* (cf. Loew<sup>33</sup>). The drugs, in moderate quantities, usually fail to relax the undrugged smooth muscle of the bronchioles, intestine, or uterus. Thus, the ability to antagonize histamine cannot be ascribed to a general antispasmodic action. Staub<sup>55</sup> and Halpern<sup>20</sup> emphasized the fact that, with 929F, 1571F, Antergan, and related compounds, there was no close correlation between the antihistamine action and the antispasmodic effects. Benadryl does exert a weak antispasmodic effect on intestinal muscle.<sup>38, 58</sup> However, Neoantergan and Pyribenzamine are practically devoid of antispasmodic action. In fact, doses of moderate size may even induce a spasm of uterine or intestinal muscle, *in vitro* and *in vivo*.<sup>13, 20, 29, 41, 55, 62</sup>

(3) *Uterine Spasm*. Nearly all the antihistamine drugs have been demonstrated to antagonize the spasmogenic action of histamine on the uterus in several animal species either *in vitro* or *in vivo*.<sup>13, 20, 29, 41, 53, 55</sup> No data are available, however, which concern the relative degree of action of these drugs in antagonizing other spasmogenic agents such as acetylcholine, barium, and pituitrin. Thus, the degree of antihistamine specificity related to uterine responses remains undetermined.

There has been no demonstration that antihistamine drugs decrease the spontaneous motility and tonus of the uterus, which means that no antispasmodic action has been demonstrated. On the other hand, contraction of uterine muscle has been induced by nearly all of the ethylenediamine derivatives, either when low dilutions were used on isolated uterine tissue<sup>13, 20, 29, 41, 55</sup> or when doses of 3.0 mg./kg. were injected intravenously in dogs.<sup>58</sup>

It may be concluded from the limited amount of data available, and the absence of contrary evidence, that proper doses of antihistamine drugs prevent the spasmogenic action of histamine on the uterus without inducing a

concomitant antispasmodic action. Thus, the antihistamine action is not dependent on a general antispasmodic action, especially since most ethylenediamine derivatives, in moderate or large doses, actually exert spasmogenic action.

Further studies with uterine muscle are needed to determine whether antihistamine drugs are relatively specific in antagonizing the oxytocic action of histamine. To the author's knowledge, none of the antihistamine drugs developed in recent years have been tested to determine whether they are capable of antagonizing the spasmogenic effects of pituitrin on uterine and other types of smooth muscle. Studies with pituitrin would extend the knowledge concerning antihistamine specificity and would either support or detract from the degree of specificity established thus far. The natural occurrence of pituitrin in the body is a cogent reason for determining whether widely used antihistamine drugs are capable of altering the role which pituitrin may play in the bodily economy.

#### *B. Vascular Effects of Histamine As Measured by Changes in Blood Pressure*

The precipitous, transient, decreased blood pressure induced with small intravenous doses of histamine and acetylcholine is largely due to arteriolar dilatation accompanied by some capillary dilatation. It is pertinent to note that the depressor effect of small doses of histamine is largely due to a *relaxing* effect upon vascular smooth muscle and that this relaxing effect of histamine is diminished by antihistamine drugs. It has been demonstrated that the depressor responses elicited by small doses of histamine were diminished but seldom annulled by Antergan,<sup>20, 40, 47</sup> Neoantergan,<sup>5, 6, 8, 47, 53</sup> Benadryl,<sup>38, 40, 53, 57</sup> Pyribenzamine<sup>40, 41, 53, 60</sup> and its thiophene analogue,<sup>29</sup> and Thephorin.<sup>30</sup> Comparative studies made by Marsh and Davis<sup>40</sup> and by Sherrod, Loew, and Schloemer<sup>53</sup> revealed that Benadryl was no less effective than Antergan, Neoantergan, and Pyribenzamine in antagonizing the depressor action of histamine in dogs. Furthermore, a comparison of the doses of drugs used by various workers to diminish the depressor effect of histamine in dogs or cats does not reveal evidence of any pronounced difference in potency among the antihistamine drugs considered.

Pertinent to the present discussion is the fact that certain antihistamine drugs also diminish the depressor effect of acetylcholine. Furthermore, several types of antihistamine drugs enhance the pressor effects of epinephrine, whereas other types either diminish or actually block and reverse the pressor effects of epinephrine. Thus, it is at once apparent, from studies based upon vascular reactions, that antihistamine drugs are not absolutely specific. It is important, therefore, to consider the relative degree of antihistamine specificity and also the information relating to one or more other drug effects which might be somewhat related to the antagonism of the vascular actions of histamine.

In general, the dose of each antihistamine drug (1.0 to 5.0 mg./kg., *i.v.*) most frequently used to demonstrate diminished depressor responses to histamine in anesthetized animals has usually been sufficient to affect the responses to either acetylcholine or epinephrine. This fact suggests a rela-

tively low degree of antihistamine specificity as measured by blood pressure changes, although, admittedly, there have been very few studies<sup>38</sup> in which gradation of doses has been employed to determine the minimum amount of antihistamine drug required to alter responses to substances other than histamine.

Sufficient data are available to demonstrate that diminution of the depressor effect of histamine after treatment with antihistamine drugs is not always coincident with, and therefore not dependent upon, one or more other phenomena. Some of the pertinent facts are indicated in TABLE 2.

Antergan and Pyribenzamine both diminished depressor responses to histamine, but this effect can scarcely be dependent upon an atropine-like action, since the depressor effects of acetylcholine were not diminished. Results with Pyribenzamine<sup>40, 41, 53, 60</sup> are typical of those obtained with its thiophene analogue<sup>29</sup> and with Neoantergan.<sup>5, 6, 8, 53</sup>

TABLE 2  
EFFECTS OF ANTIHISTAMINE DRUGS ON VASCULAR RESPONSES IN ANESTHETIZED DOGS AND CATS

Compound	Depressor effect of histamine diminished	Depressor effect of acetylcholine diminished	Pressor effect of epinephrine		
			enhanced	diminished	blocked or reversed
Antergan·HCl	+	—	+	—	—
Pyribenzamine·HCl	+	—	+	—	—
Benadryl·HCl	+	+	+	—	—
929F	+	+	—	+	—
Theporin tartrate	+	+	—	+	—
$\alpha$ -Naphthylmethylethyl- $\beta$ -chloroethylamine·HCl	+	+	—	+	+

\* Only a weak atropine-like action has been demonstrated.

Doses of Benadryl (1.0 and 3.0 mg./kg., *i.v.*), used to demonstrate the diminution of depressor responses to histamine, were also effective in reducing but not abolishing the depressor response to acetylcholine.<sup>38</sup> The anti-acetylcholine action of Benadryl, as demonstrated by blood pressure responses, was relatively weak, since it required 1/100 as much atropine to duplicate the effect.<sup>38</sup> Other chemical types of antihistamine drugs, as represented by 929F, Theporin, and  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine, do not exert any pronounced atropine-like effects on the vascular system.

Thus, only a low degree of atropine-like action has been demonstrated with some antihistamine drugs, and the ethylenediamine compounds such as Antergan, Neoantergan, and Pyribenzamine were practically devoid of such action. Even when present, the atropine-like action is weak and certainly does not account for the antagonism of histamine, since one has only to recall that the action of histamine is frequently demonstrated and quantitated by using atropinized test objects of high sensitivity, *i.e.*, by measur-

ing contractions of the atropinized, isolated intestine or by measuring depressor responses in the atropinized cat.

In summary, these comparisons of vascular responses indicate that ethylenediamine derivatives of aniline (Antergan) and  $\alpha$ -aminopyridine (Neoantergan, Pyribenzamine, and its thiophene analogue) exert antihistamine action without antagonizing the depressor effects of acetylcholine. Benadryl exhibited less specific effects related to blood pressure responses, since the depressor responses to both histamine and acetylcholine were diminished, although the atropine-like action of Benadryl was no more than 1/100 that of atropine itself. The antiacetylcholine action of  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine and Thephorin was weak, or not demonstrated, with doses employed to antagonize histamine, although more data relating to these compounds are needed before drawing conclusions concerning degree of atropine-like action exerted. It is very unlikely that the ability to antagonize the depressor effect of histamine is dependent to a significant degree upon an atropine-like action. When such action is readily demonstrable, however, it detracts from the degree of antihistamine specificity.

The ability of antihistamine drugs to alter the pressor effects of epinephrine must also receive consideration with respect to the problem of specificity and in relation to the mode of histamine antagonism. As indicated in TABLE 2, it has been demonstrated that Antergan,<sup>12, 46</sup> Neoantergan,<sup>53</sup> Pyribenzamine,<sup>53, 60</sup> and Benadryl<sup>38, 53</sup> enhance the pressor response to small doses of epinephrine in anesthetized animals. Comparable results have been obtained with the thiophene analogue of Pyribenzamine<sup>29</sup> and quaternary derivatives of Benadryl.<sup>54</sup> Although the enhanced pressor response to epinephrine has frequently occurred concomitantly with the diminished depressor response to histamine, there are definite reasons for believing that histamine antagonism is not dependent upon the enhanced pressor response to epinephrine.

First, small doses of Benadryl antagonized histamine without altering the response to epinephrine.<sup>38</sup> In cats, Yonkman *et al.*<sup>61</sup> injected small doses of Pyribenzamine, which sufficed to antagonize histamine but which frequently failed to alter responses to epinephrine.

Secondly, several compounds which diminish the depressor effects of histamine not only fail to enhance the pressor effect of epinephrine but actually diminish the pressor effects (TABLE 2). Thus, 929F and Thephorin antagonize histamine, Thephorin being quite active, and both drugs diminish the pressor response to epinephrine.<sup>7, 11, 30</sup>

Even more striking is the demonstration<sup>2</sup> that  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine strongly antagonized the depressor effect of histamine in dogs and completely reversed the pressor response of epinephrine to a depressor response. The ability of this naphthyl compound to diminish the depressor response to histamine and to reverse the pressor effect of epinephrine is illustrated by FIGURE 2.

In summary, data relating to alterations in blood pressure reveal that all drugs considered which diminished the depressor response to histamine were capable of altering the blood pressure to epinephrine. However, some

of the drugs which antagonize histamine enhanced the pressor effect of epinephrine, whereas others either diminished or actually reversed the pressor response to epinephrine. It is therefore difficult to conceive that either enhancement or reversal of the pressor response to epinephrine could account for the antagonism of histamine. Furthermore, small doses of Benadryl and Pyribenzamine antagonized histamine without significantly altering the pressor response to epinephrine. This is further evidence that



FIGURE 2. The effect of  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine hydrochloride on vascular responses to histamine and epinephrine. Record of carotid blood pressure of dog under sodium pentobarbital anesthesia. Above: Control responses. Depressor effect of histamine diphosphate (50 $\gamma$ ) and pressor effect of epinephrine hydrochloride (20 $\gamma$ ). Below: Record taken 10 to 20 minutes after intravenous injection of the drug in a dose of 3.0 mg./kg. Note blocking of the depressor action of histamine and reversal of the pressor action of epinephrine.

the histamine antagonism was not necessarily dependent upon a mechanism which involved enhancement of epinephrine effect. The failure of small doses of Benadryl and Pyribenzamine to alter responses to epinephrine also reveals some specificity of antihistamine action, especially with Pyribenzamine, since it also failed to exert an antiacetylcholine action.

### C. Localized Cutaneous Responses to Histamine

The experimental studies concerning histamine-antihistamine antagonism related to capillary dilatation and increased capillary permeability are almost wholly concerned with observations and measurement of the cutaneous

responses to histamine. The effectiveness or noneffectiveness of drugs in diminishing localized cutaneous reactions to a variety of stimuli provides information concerning their antihistamine specificity. Following intradermal injection, various substances induce a localized edema or wheal response, which is due, for the most part, to increased capillary permeability which is quite independent of nervous factors. The accompanying flare, on the other hand, is largely dependent upon vasodilatation effected through axon reflex action. Analysis of the wheal and flare response, as influenced by antihistamine drugs, must include consideration of influences on increased capillary permeability, vasoconstriction, vasodilatation, and the nervous factors related to the flare reaction.

It is not likely that changes in vascularity *per se* induced by antihistamine drugs would account for diminution of the wheal and flare response to histamine or other substances, since neither vasodilator nor vasoconstrictor action of definite degree has been demonstrated with antihistamine drugs. Furthermore, as will be more apparent in the later discussion, if a vasodilator or vasoconstrictor action accounted for diminution of histamine effects, then cutaneous responses to numerous other agents should be diminished, which is not the case.

Apparently, all antihistamine drugs exert some degree of local anesthetic action.<sup>9, 11, 12, 20, 27, 28, 37, 41, 50, 55</sup> Injection in localized areas or topical administration of suitable quantities could affect cutaneous reactions by virtue of local anesthetic action. Friedlaender and Feinberg<sup>18</sup> have completely ignored the probability that local anesthetic action of Benadryl (5 per cent) applied topically to scratched surfaces of human skin accounted, in part, for the reduced flare and wheal responses to histamine, codeine, and allergens. Local anesthesia is known to block the axon reflex and thus diminish the flare reaction. Diminished vascularity, in turn, might indirectly diminish the wheal response. Experiments made by Leavitt and Code<sup>27, 28</sup> demonstrated several important facts related to the properties of Benadryl and the interpretation of its effect on cutaneous reactions. Intradermal injections of Benadryl in man induced an appreciable degree of cutaneous anesthesia. In addition, cutaneous responses to histamine were definitely diminished by both Benadryl and Procaine.<sup>28</sup> After the local anesthetic effect of Benadryl and Procaine had disappeared, however, some other action of Benadryl persisted and diminished the cutaneous reaction to histamine. Thus, the local anesthetic action of Benadryl did not account for the persistent inhibitory influence on the cutaneous reaction to histamine and it is probable that a direct antagonism of histamine was also involved.

Aaron and Abramson<sup>1</sup> introduced Pyribenzamine and Procaine into human skin by iontophoresis one and one-half hours before introducing histamine. Pyribenzamine inhibited the histamine wheal and flare reaction, and, since Procaine failed to do so, one can assume that the positive results with Pyribenzamine were referable to an antagonism of histamine and not to a local anesthetic action, unless it is shown that such action is exceedingly prolonged. These experiments with Benadryl and Pyribenzamine certainly emphasize the necessity of considering local anesthetic action when inter-

preting the influence of antihistamine drugs on cutaneous responses, especially when drugs are applied topically or injected locally. The author is not aware of any direct evidence that systemic anesthetic action is induced by oral administration of moderate quantities of antihistamine drugs. However, oral doses frequently diminish cutaneous reactions to histamine (see Loew,<sup>38</sup> page 558). The evidence cited enhances the probability that a direct antagonism of histamine accounts for the fact that antihistamine drugs diminish cutaneous responses to histamine.

Other evidence eliminates the possibility that an atropine-like action of antihistamine drugs accounts for the alteration of cutaneous responses and also provides support for belief in antihistamine specificity. In rabbits, Last and Loew<sup>28</sup> measured the degree of increased capillary permeability in the abdominal skin by observing the extent to which intravenously injected Trypan Blue dye extravasated into areas receiving intradermal injections of histamine and other substances. Positive Trypan Blue responses, indicative of increased capillary permeability, were readily induced with histamine. Pretreatment of the animals with Benadryl or Neoantergan, by parenteral administration, diminished or eliminated the increased capillary permeability due to histamine as indicated by diminished or abolished Trypan Blue responses. The increased capillary permeability induced with trypsin, snake venom, staphylococcus toxin, tetracaine (Pontocaine), and codeine was not altered by treatment with Benadryl, or by Neoantergan when the latter was used. The failure of antihistamine drugs to alter responses to these substances suggests that liberated histamine did not cause the increased capillary permeability. Prevention of responses to histamine could scarcely be due to any atropine-like action of antihistamine drugs, since Neoantergan was very effective even though nearly devoid of atropine-like action. Furthermore, atropine itself does not alter this type of cutaneous reaction in man.<sup>56</sup> Because of its antiacetylcholine specificity, one would not expect it to alter cutaneous responses to histamine, since it is improbable that any choline esters are involved in the reaction. Neither Mecholyl<sup>28</sup> nor acetylcholine<sup>48</sup> induced Trypan Blue reactions in rabbits.

If the ability of antihistamine drugs to diminish Trypan Blue reactions to histamine were related to vasodilator or vasoconstrictor actions of the drugs, the responses to the other substances should have been altered also. Added consideration must be given to the possibility that vasoconstriction aids in diminishing the Trypan Blue response to histamine because of the previously mentioned fact that several antihistamine drugs enhance the pressor (vasoconstrictor?) effect of epinephrine. We have repeated the experiments in rabbits<sup>26</sup> and determined that the Trypan Blue responses to histamine were readily annulled by  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine in doses which were known to be adequate to diminish several effects of histamine in other animal species and which blocked the pressor action of epinephrine in rabbits. It was noted also in these experiments that the naphthyl derivative greatly diminished the blanching due to vasoconstriction in areas injected intradermally with epinephrine. Thus, the ability of histamine to increase capillary permeability was annulled by a drug which antagonized

histamine and the vasoconstrictive action of epinephrine. This evidence, in conjunction with other considerations, supports the thesis that vasoconstriction does not account for the ability of antihistamine drugs to prevent histamine from increasing capillary permeability.

Thus, an analysis of the experimental evidence cited, as well as a review of other pertinent information,<sup>33</sup> leads to the belief that the ability of antihistamine drugs to diminish the cutaneous flare and wheal response to histamine is largely due to a specific blocking of the action of histamine. Histamine-induced vasodilatation and increased capillary permeability are diminished, and these effects cannot be due entirely to local anesthetic, atropine-like, or vasoconstrictive actions of antihistamine drugs. The local anesthetic action of several antihistamine drugs is sufficiently strong to diminish cutaneous reactions to histamine and probably to other agents, at least when the drugs are applied topically or injected locally.

### *Discussion*

Analysis of the available evidence reveals that antihistamine drugs such as Antergan, Antistine, Neoantergan, Benadryl, Pyribenzamine, and the thiophene analogue of the latter are capable of diminishing the ability of histamine to contract smooth muscle of several organs, to relax vascular smooth muscle, and to increase permeability of capillary endothelium. The gastric secretagogue action of histamine is not blocked, and there are insufficient data to prove that the secretagogue action on other cells is affected.

The prominence of the drug antagonism of the contracting and relaxing effects of histamine on smooth muscle and the increase of capillary permeability suggest that a relatively specific antihistamine action is involved. Other pharmacological actions have been demonstrated with antihistamine drugs, but the foregoing analysis reveals that experiments of proper design yield evidence which indicates that none of these actions can account for any more than a small part of histamine antagonism. The evidence supports the thesis that the drugs discussed are relatively specific antihistamine drugs or antihistaminics. In order to avoid confusion in this rapidly developing phase of pharmacology, it is recommended that new drugs should not be classified as antihistaminics unless a moderate degree of antihistamine specificity has been demonstrated.

Several  $\beta$ -chloroethylamine derivatives, chemically related to Dibenzamine,<sup>45</sup> have been demonstrated to be moderately or highly effective in diminishing several effects of histamine.<sup>2, 33, 39</sup> At present, however, the author is reluctant to class these compounds as antihistaminics in view of their marked ability to induce epinephrine reversal and to diminish, block, and reverse some responses to adrenergic nerve stimulation. Parenthetically, the author wishes to admit that confusion may stem from the statement that higher alkyl homologues of the  $\beta$ -chloroethylamines recently studied were devoid of histamine antagonistic properties.<sup>2, 33</sup> In contrast to lower alkyl homologues, they did fail to prevent histamine-induced bronchioconstriction in guinea pigs, but recent experiments indicate that all the compounds, in-



cluding the higher alkyl homologues, are capable of diminishing and blocking the depressor effects of histamine in anesthetized dogs.<sup>34</sup> Extension of these studies may establish the fact that certain compounds can preferentially select a given tissue and antagonize the effects of histamine thereon. Recognition of this possibility should suffice to warn that a single type of pharmacological test might fail to reveal an ability to antagonize some effect of histamine.

The very variety of pharmacological effects which is elicited to some degree by antihistamine drugs investigated thus far suggests that even other types of action may yet be demonstrated and that future antihistamine drugs may possess various combinations of pharmacological properties. In fact, the demonstration of histamine antagonism possibly should be regarded as evidence that the drugs in question have some special affinity for certain components of effector cells. Slight qualitative differences between the natures of such affinities may determine whether the drugs exert prominent antihistamine action or some other blocking or stimulating action on muscle, nerve, secretory, or endothelial cells.

The varied but somewhat minor pharmacological actions of antihistamine drugs which detract from the degree of specificity may be of more importance than realized, since they could conceivably throw light on the locus and mode of action of these drugs. It may not be mere coincidence, for example, that the several chemical types of drugs which antagonize histamine all either enhance or diminish certain effects of epinephrine, and that all exert some antiacetylcholine and local anesthetic action. These varied actions may be dependent upon some common mechanism or several closely related ones.

### *Summary*

(1) Antihistamine drugs diminish or block the most prominent actions of histamine, except the gastric secretagogue action. Data relating to other secretory effects are needed.

(2) The drugs exert a relatively high degree of specific antihistamine action on smooth muscle and capillaries, since, under proper conditions, this action is readily demonstrable with small doses and is usually nondependent on local anesthetic, antispasmodic, spasmogenic, sympathomimetic, or sympatholytic action.

(3) It is very unlikely that antihistamine action on any tissue is referable to either an enhancement or diminution of the excitatory effects of epinephrine, since several potent histamine antagonists enhance the pressor response to epinephrine, whereas others strongly antagonize histamine although they diminish and even block and reverse the pressor effects of epinephrine.

(4) When antihistamine drugs, even though relatively specific, are used as research tools or diagnostic aids, due consideration must be given to a variety of pharmacological actions when experiments are planned and the findings are interpreted. For example, high local concentration of these drugs following topical application or local injection can induce a degree of local anesthesia capable of diminishing cutaneous responses to histamine and probably to other agents.

(5) When measured under certain conditions, the potency of Benadryl is less than that of Neoantergan and Pyribenzamine. The antihistamine specificity of Benadryl may therefore be less, but consideration must also be given to the fact that, in some circumstances, no differences in potency have been demonstrated. Furthermore, each of these compounds enhances the pressor action of epinephrine, and the antispasmodic action of Benadryl is replaced by a weak spasmogenic action in all the ethylenediamine derivatives. There has been no demonstration of a pronounced variation in the degree of local anesthetic action induced by antihistamine drugs.

#### *Addendum*

A majority of the side effects in human beings by antihistaminics are probably unrelated to a specific histamine blocking action. Furthermore, more recent evidence of the effectiveness of certain antihistaminics in controlling symptoms of motion sickness and Parkinsonism suggests drug activities other than blockade of histamine. It is probable that future studies with compounds now regarded as highly specific antihistaminics will reveal other nonspecific actions of theoretical and practical interest.

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# ANTISTINE AND RELATED IMIDAZOLINES

By Rolf Meier

*Medical Director, Ciba Limited, and University of Basle, Basle, Switzerland*

The series of aromatic imidazolines which have been investigated for about ten years in our laboratories has offered special possibilities for the systematic variation of pharmacological effects. This is due to the fact that, in this group, substances are found with a specific effect in several directions.

The histaminolytic efficiency of numbers of the aromatic imidazolines is only an example of this specificity of effect. One of the most effective substances in this direction is Antistine<sup>1</sup> (formula, see page 1163). Its structural formula is similar to that of Antergan. A series of imidazoline derivatives possess, likewise, a high histaminolytic effect, though small changes in the structure are sufficient to reduce strongly this efficacy.

Antistine shows, in comparison with the other histaminolytics, several special properties. The toxicity of all histaminolytically effective substances is about of the same order. However, the dose with which the first side-effects are observable is different. According to our experience, this dose is relatively higher in the case of Antistine than for other substances. Also, the tolerability on local application seems to be better. Antistine, as an antihistaminic agent, is less active than Pyribenzamine,<sup>2</sup> Neoantergan,<sup>3</sup> and the Phenothiazine<sup>4</sup> derivatives. This is as true for the reaction of isolated organs as for the antagonistic effect on histamine toxicity in the animal. The difference varies, depending on the object. On the other hand, the action on the anaphylactic shock is not less but is of the same order of potency as that of the most effective antihistaminics (TABLE 1). Besides these effects, Antistine differs from the other antihistaminics in its non-antihistaminic pharmacological actions. For example, it has no pronounced antiacetylcholine nor adrenaline reinforcing effect; on the contrary, it has a slight sympathicolytic action on different test objects (FIGURE 1).

These differences show that Antistine possesses a complex of pharmacological effects of its own. They show further that pronounced antianaphylactic activity may be produced without any marked effect on the autonomic system (as has been demonstrated already by Loew). On the other hand, the antihistaminic effect seems to be correlated, in many different chemical structures, with the effects on the autonomic nervous system. This seems to indicate that those chemical compounds potentially possess the other specific features.

It seemed to be interesting from the theoretical and practical point of view to investigate whether substances could be devised from the same basic structure, possessing only the one or the other of those specific effects. In the series of aromatic imidazolines synthesized by Hartmann and Isler and Miescher, Urech, and Klarer, we established examples to elucidate this possibility. In the series of benzimidazolines, it was already striking that,

by small changes of the molecule, strongly effective sympathicomimetics could be converted into sympathicolitics (see FIGURE 2.)

Here, it is perhaps noteworthy that the sympathicomimetic, 2020/n, and some other similar substances of this series have approximately a ten to one hundred times stronger sympathicomimetic effect on the vessels and on the blood pressure than epinephrine or norepinephrine. In view of the characterization of the sympathicomimetic receptors, this fact has a certain interest.<sup>10</sup>

TABLE 1  
COMPARISON OF ANTIHISTAMINIC AND ANTIANAPHYLACTIC ACTION OF ANTIHISTAMINIC DRUGS ON DIFFERENT TEST OBJECTS

Preparations	Toxicity Rabbit i.v. (L.D. 50)	Spasmolysis histamine- test Guinea- pig ( $5 \times 10^{-8}$ )	Protection in vitro Schultz- Dale	Protection in vivo Guinea- pig against anaphyl death	Number of lethal histamin- doses i.v. Guinea- pig (20 mg./kg. cc.)
Antergan .....	$2 \times 10^{-5}$	$2.5 \times 10^{-8}$	$2.5 \times 10^{-8}$	$5 \times 10^{-7}$	120
Neo-antergan .....	$1.9 \times 10^{-5}$	$2 \times 10^{-9}$	$10^{-8}$	$5 \times 10^{-6}$	300
Pyribenzamine .....	$1.4 \times 10^{-5}$	$2 \times 10^{-8}$	$2 \times 10^{-8}$	$3 \times 10^{-6}$	150
Antistine .....	$1.8 \times 10^{-5}$	$10^{-7}$	$10^{-7}$	$3 \times 10^{-6}$	30
Phenothiazine II 3277 R.P..	"/.	$5 \times 10^{-8}$	"/.	$10^{-6}$	1500

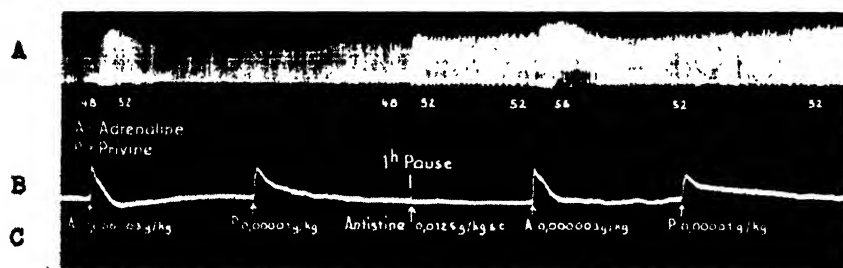


FIGURE 1. Comparison of the pressor effect of adrenaline and Privine, before and after s.c. Antistine treatment. No adrenaline reinforcing effect. A—Respiration; B—Blood pressure; C—Time 6".

Still greater than the series of benzylimidazolines are the possibilities of variation in the series of aromatic imidazoline derivatives of aniline. Antistine belongs to this series. Also, strongly effective sympathicomimetics such as Otrivine have been found. Only lately, one of the strongest effective sympathicolitics has been detected in this series.

The basic structure in this series shows a clearly defined differentiation which evidently determines the change in specific effect. Nuclear substitutes can influence, in more or less every case, the intensity of the specific efficiency. This shows that, in this series, compounds, in spite of great chemical similarity, react in a fundamentally different way with the cell substrate.

For the characterization of the specificity of effect, the question is raised, first of all, whether this specificity is complete in every case. For the sympatholytic and antihistaminic action, the comparison with a physiological humoral factor of the body does not permit a decision of this question. Only for the sympathicomimetic effect does a comparison with the physiological agent, epinephrine or norepinephrine, give this information.

The classification of the effect according to the nature and intensity, for a series of different objects, shows that the sympathicomimetic imidazolines act analogously to adrenaline upon a whole series, but not on others.

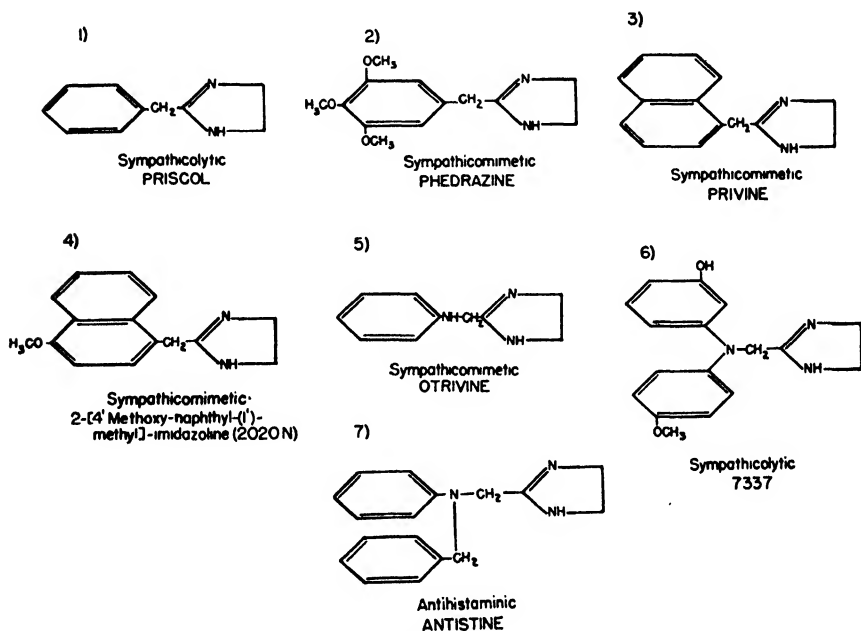


FIGURE 2.

The vasoconstrictive effect of Otrivine and also of Privine on the isolated vessels and on blood pressure is of the same order as that of epinephrine (FIGURE 3). On the isolated heart, the coronary dilatation of adrenaline is missing just as is the effect on amplitude and frequency. Therefore, a different action on the circulation, especially the perfusion of the different peripheral areas, occurs as previously published.<sup>8</sup>

The effect on the isolated seminal vesicles and the isolated intestine of the rabbit is similar to that of adrenaline—stimulating in the first and relaxing in the latter. On the isolated intestine of the guinea pig, the action of the imidazoline is contrary to that of adrenaline. This sympathicomimetic effect consequently favors only a selected number of sympathetic points of attack.

A characterization of the sympatholytic and histaminolytic effects of imidazolines can result from their comparison with other analogous sub-

stances or investigations of the antagonistic effect towards histamine, adrenaline, acetylcholine, *etc.*

The comparison of the effects of sympathicolytic and histaminolytic imidazoline derivatives with those of the primary effective substances, such as adrenaline and histamine, shows that there is, in general, a typical contrary action on most of the objects, namely, on the blood pressure, on the in-

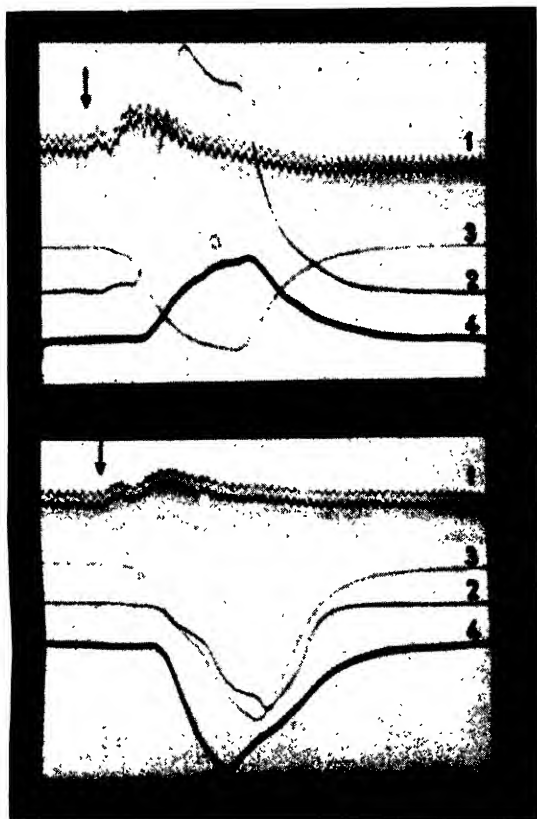


FIGURE 3. Effect of adrenaline and Privine on the blood pressure and on the perfusion of different arterial areas on the cat. 1—blood pressure of the carotid artery; 2—perfusion of the mesenteric artery; 3—perfusion of the renal artery; 4—perfusion of the femoral artery. A—adrenaline 2 $\gamma$ /kg. i.v.; B—Privine 5 $\gamma$ /kg. i.v. Change of records of arterial perfusion: upwards—increase; downwards—decrease.

testine, and on the seminal vesicles. On isolated objects, however, a similar effect occurs exceptionally, independent of whether a sympathicolytic or a histaminolytic effect is also present. These differences may be attributed partly to the different physiological autonomic equilibrium of the organs tested but could not be interpreted generally in this way.

The specific antagonistic effect of those drugs has to be clarified by testing the antagonistic action on different organs. The results of this investigation indicate that, in each group of imidazolines with specific effect, a series of antagonisms of high specificity could be demonstrated (FIGURE 4).

Methods	Animals	Application	Measured effective dosage of		Histamine
			Oral	IV	
Toxicity LD <sub>50</sub>	Mice	LD	25-40	35-50	10 <sup>-6</sup>
Lesions	Mice	intraperitoneal	10 <sup>-7</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
	Guinea-pigs	intraperitoneal	10 <sup>-5</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
Heart	Mice	intraperitoneal	10 <sup>-7</sup>	10 <sup>-7</sup>	10 <sup>-7</sup>
Speed of onset of convulsions	Guinea-pigs	intraperitoneal	10 <sup>-3</sup>	3-10 <sup>-4</sup>	10 <sup>-7</sup>
	Mice	LD / SC	10 <sup>-9</sup>	10 <sup>-7</sup>	10 <sup>-5</sup>
Blood pressure	Mice	intraperitoneal	10 <sup>-7</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>
	Guinea-pigs	intraperitoneal	10 <sup>-6</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>
Intestine	Mice	intraperitoneal	10 <sup>-7</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>
	Guinea-pigs	intraperitoneal	10 <sup>-6</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>
Skeletal muscle	Mice	intraperitoneal	10 <sup>-7</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>
	Guinea-pigs	intraperitoneal	10 <sup>-6</sup>	10 <sup>-5</sup>	10 <sup>-5</sup>

[illegible]

FIGURE 4. Comparison of action of different imidazoline derivatives with the effect of adrenaline, acetylcholine, and histamine and of the antagonistic effect to these substances.



The examination, particularly the quantitative evaluation of antagonistic reactions, presents various difficulties. They consist of the special conditions which have to be ascertained according to the relative dose and the size of the dose evaluated for each test organ. Another difficulty is the fact that, on certain organs, no antagonistic effects occur. Thus, the possibilities of evaluation are limited in many respects.

If a typically suitable test object is chosen (e.g., the reaction of the isolated vessels), the relatively high specificity of the antagonistic reaction can be easily demonstrated. For example, the antagonism of the sympathicolytic for adrenaline is in proportion to Antistine and Otrivine in the ratio of 100:1:0, or the antagonism of Antistine for histamine is in proportion to both the others as 100:1:0; the relations are the opposite in these cases. As to the reaction on the seminal vesicles, the specificity is present in the same way. The relative proportion of effect is somewhat different; thus, for example, adrenaline antagonism, sympathicolytic 7337: Otrivine: Antistine = 100:3:1 and the histamine antagonism of Antistine: sympathicolytic 7337: Otrivine = 100:1:0.1.

Nearly the same relation exists for the reaction of the isolated guinea-pig intestine. On other objects, the relative effectiveness of the two compounds shows other differences. The histamine effect on the isolated heart is counteracted by both substances in the same concentration. Regarding blood pressure, Antistine does not alter the adrenaline effect. The sympathicolytic acts in the same antagonistic concentration as on other organs. These few examples show that, again, the antagonistic effect, specific in principle, is typical only on certain organ systems.

The investigation of the antagonistic action against acetylcholine demonstrates that none of these substances has a specific antagonistic effect but that occasionally such may be present. The isolated vessels, dilated by acetylcholine, are constricted by Antistine, in spite of the fact that it has no action by itself. The sympathicolytic agent has no effect even in high concentrations. Both substances act antagonistically to the same degree on the seminal vesicles.

These differences of antagonism are independent of the primary effect upon the normal function, which exists without the previous influence of the agonists. This shows that the specific effect of the antagonists appears only when a potential action of the agonists exists. Consequently, these antagonistic substances will exert their specific action only when a specific primary effect is present; otherwise, their "unspecific" effects will appear. In principal, these belong in the same class as the primary effects or they are unspecifically antagonistic.

The demonstration of an effect of a high degree of specificity on individual objects in no way excludes the absence of this effect or the presence of unspecific actions on other objects. The dosage range between these specific and nonspecific effects is not always large enough to exclude the fact that these unspecific effects are not involved in the therapeutic phenomenon or the side reactions.

These results show that the group of aromatic imidazolines contains substances with high specificity, but they show also that every group has a

specific type of action when compared with other members of the same pharmacological group. This can be shown for the sympathicolytic effect in a way similar to that done with the sympathicomimetic and antihistaminic effect (*i.e.*, by comparison with other sympathicolytic agents), as we have already published. The analysis of the circulatory effect is especially suitable. Of these substances, only one example need be given for the group

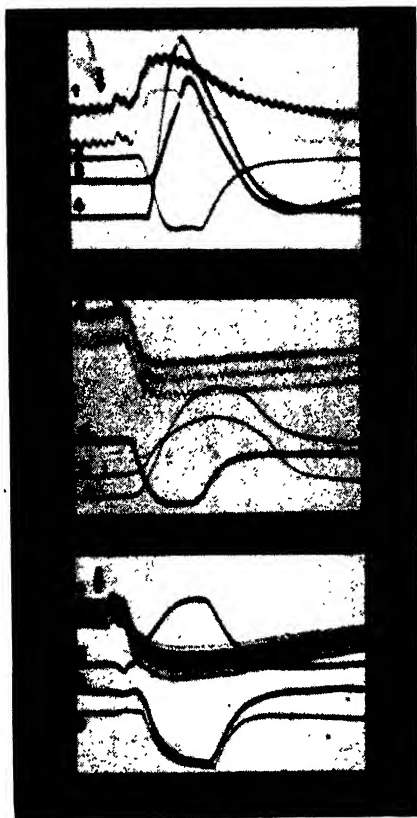


FIGURE 5. Effect of pretreatment with Prisol and sympathicolytic imidazoline 7337 on the adrenaline effect on blood pressure and the perfusion of different arterial areas on the cat. 1—blood pressure of the carotid artery; 2—perfusion of the mesenteric artery; 3—perfusion of the renal artery; 4—perfusion of the femoral artery. A—adrenaline 2 $\gamma$ /kg. i.v.; B—adrenaline 2 $\gamma$ /kg. i.v. after pretreatment with 0.010 g./kg. s.c. 7337; C—adrenaline 2 $\gamma$ /kg. i.v. after pretreatment with 0.025 g./kg. s.c. Prisol. Change of records of arterial perfusion: upwards = increase; downwards = decrease.

of sympathicolytics. In spite of the two substances, Prisol and 7337, producing an adrenalin reversal on the blood pressure, the circulatory response measured on the perfusion of different arterial areas as mesenteric, femoral, and renal artery shows reversal of blood flow reaction only in the case of Prisol—not with 7337 (FIGURE 5). So it can be seen that, in spite of typical effect in one direction, the same may be lacking in another one. Thus, most groups of specific agents seems to have an individual type of effect.

Since, in the group of the aromatic imidazoles with fundamentally similar chemical structure, both a pronounced system-restricted and an

organ-restricted differentiation of various specific effects occur as a result of small chemical changes, it is permissible to conclude that the influenced substrate must possess an analogous differentiation. It is responsible both for the systematic effect of the agonists and antagonists and the multiplicity of the possibilities of differentiation. This differentiation must be of such a type that the system can react upon substances of a quite different nature with contrary or differentiated reaction, and that, in the case of a large degree of chemical similarity of the substances, completely divergent reactions can arise.

Under these circumstances, it seemed to be of special interest to investigate whether differences in the behavior of these imidazolines could be found which are parallel to their specific effectiveness. The quantitative pharmacological analysis of the reaction with the substrate has been investigated in different directions and, I may add, without great success. In spite of that, however, the results of these investigations seem to have some general importance.

The determination of the dose-effect curves gives the reaction equilibrium. In this respect, the dose-effect curve of the primary effective substances and of the antagonistic reactions are to be considered. These should be determined on the greatest possible number of objects, where a specific or more unspecific effect is present. Various difficulties stand in the way of such investigations, which consist partly in the great number of necessary experiments, partly in the primary and antagonistic reactions not occurring in the same way, *etc.* The dose-effect curves for the primary substances—adrenaline, histamine, acetylcholine, and other substances—were ascertained, and the antagonistic effect of specifically acting imidazolines and of other selected substances towards the primary effects were likewise established. For example, we have investigated as follows:<sup>12,13,14</sup> on the seminal vesicles—adrenaline, acetylcholine, histamine, and Otrivine; on the vessels—a greater number of sympathicomimetics and acetylcholine; on the intestine—acetylcholine, histamine, Priscol, and Otrivine. All the substances tested on the same object give very similar dose-effect curves (FIGURE 6). Moreover, it is a matter of indifference whether the substances vary in efficacy by several powers of 10 or belong to quite different groups. Only occasional differences exist between certain dose-effect curves. These, perhaps, have a decisive importance. Considering the margin of error of our methods, however, it is premature to draw any conclusions.

This can signify that the specific, different substrates for the individual substances react in a very similar way towards the various substances or that an unspecific physico-chemical process of the equilibrium solution/cell determines the analogous behavior, and that the characteristic properties of the specific point of attack is concealed by these. The dose-effect curve is consequently not to be made directly responsible for the specificity of the effect. In most cases, the difference of the reaction consists exclusively in the variation of the absolute concentration present in the solution which is necessary for the production of equal effects.

The antagonistic effects have to be investigated in the same way. In general, there exists a parallelism of the antagonistic dose-effect curves and

of those of the primary effect, inasmuch as the former have, largely, a somewhat flatter course. The dosage-action curves of the specific sympatholytic agents against adrenaline and a sympathicomimetic imidazoline

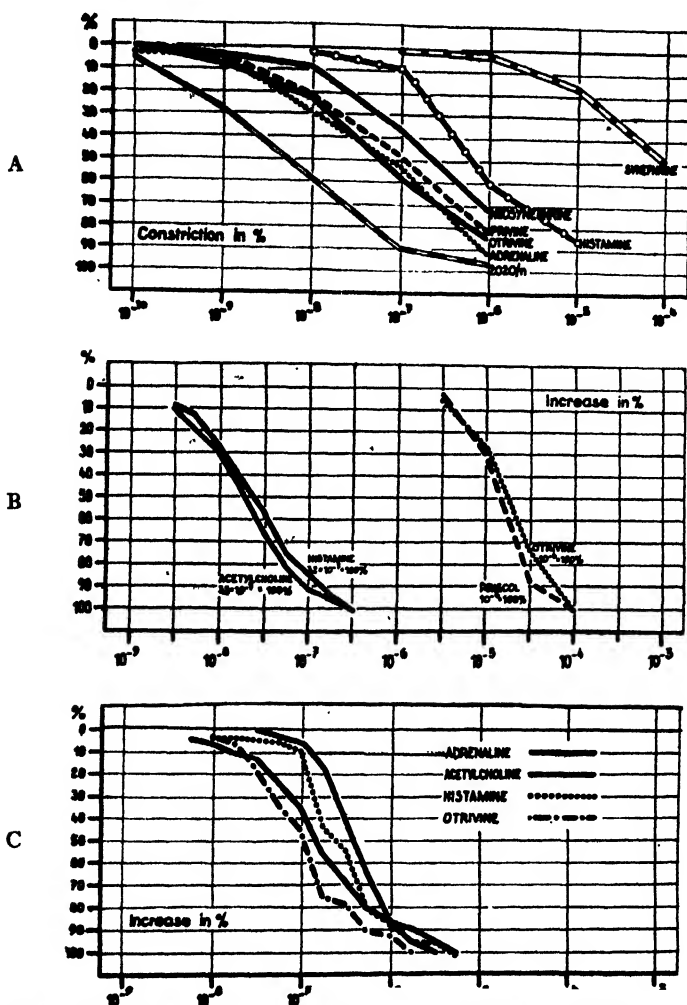


FIGURE 6. Dose-effect graphs showing the action of a series of substances with specific and nonspecific effect on perfused isolated blood vessels of the rabbit and the seminal vesicle and the isolated intestine of the guinea pig. A—action on perfused hindlegs of the rabbit (abs.—concentration of drug; ord.—inhibition of flow in percentage); B—action on isolated intestine of the guinea pig (abs.—concentration of drug; ord.—contraction in percentage of the maximal response); C—action on isolated seminal vesicle of the guinea pig (abs.—concentration of drug; ord.—contraction in percentage of the maximal response).

are all parallel, but the "unspecific action" of acetylcholine also shows the same behavior (FIGURE 7).

On the isolated intestine only the antagonistic effect on acetylcholine and histamine can be determined. The curves of the antagonistic effect of Antistine, atropine, and adrenaline to histamine have a similar shape; the

sympathcolytic imidazoline is different (FIGURE 8). The antagonistic reaction of atropine, Antistine, and the sympathcolytic imidazoline against

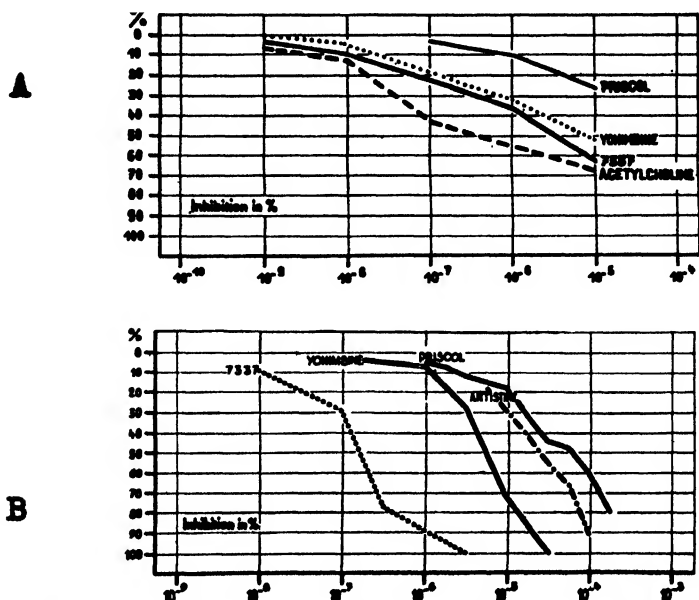


FIGURE 7. Dose-effect graphs showing antagonistic effect of different drugs to adrenaline on different test objects. A—antagonistic effect on isolated perfused hindleg of the rabbit (inhibition of adrenaline contraction—50–60 per cent of maximal response: abs.—concentration of drug; ord.—inhibition of adrenaline contraction in per cent of flow); B—antagonistic effect on isolated seminal vesicles of the guinea pig (inhibition of maximal response with threshold dose of adrenaline in per cent of maximal adrenaline effect: abs.—concentration of drug; ord.—inhibition of adrenaline contraction in percentage).

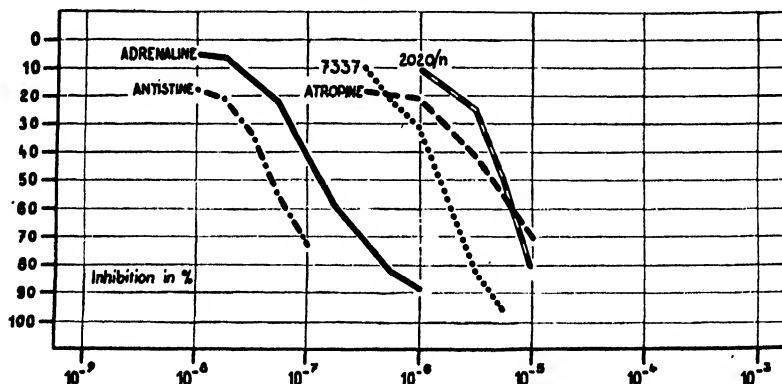


FIGURE 8. Dose-effect graph showing antagonistic effect of different drugs to histamine on the isolated intestine of the guinea pig. Inhibition of maximal response with threshold dose of histamine in per cent of maximal histamine effect: abs.—concentration of drug; ord.—inhibition of histamine contraction in percentage.

acetylcholine is similar; only the adrenaline curve shows a different shape (FIGURE 9). The reaction on the seminal vesicle also shows no certain difference between the curve of the specific sympathcolytic and other

unspecific substances against adrenaline. Certainly these examples are only a few out of the number possible and perhaps necessary. In general, however, the results may justify the assumption that there exists no simple connection between the dosage-effect curve and the specificity of the antagonistic effect.

A second possibility for the characterization of the type of reaction consists in the determination of the time-effect relation. If there are definite differences in the time where maximal effects are produced, these could also be responsible for the strength of the effect.

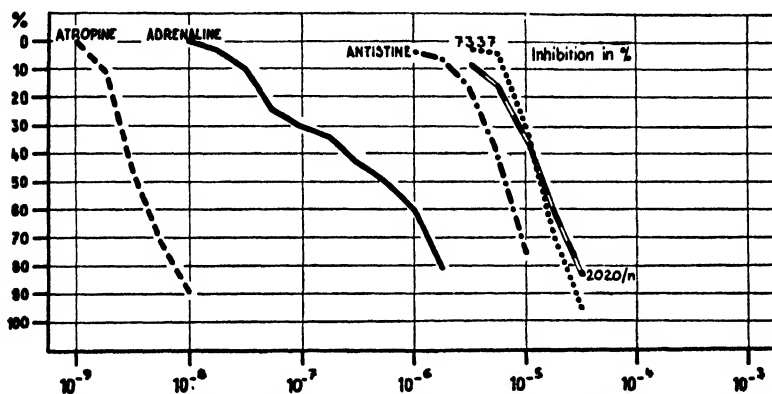


FIGURE 9. Dose-effect graph showing the antagonistic effect of different drugs to acetylcholine on isolated intestine of the guinea pig. Inhibition of maximal response with threshold dose of acetylcholine in per cent of maximal acetylcholine effect; etc., concentration of drug; and—inhibition of acetylcholine contraction in percentage.

In such experiments, the penetration velocity naturally plays a significant part. The penetration velocity is likewise a characteristic of the reaction of the various substances which have importance for the specific equilibrium, *etc.*, without being necessarily of direct importance to the specific effect. Technically, the antagonist is added to the test object and subsequently the agonistic compound at various intervals (FIGURES 10 and 11). In all the experiments, concentrations were chosen so that the agonistic substance produced a full contraction, while the antagonist abolished this by approximately 60–70 per cent. Despite the fact that it resulted in a series of differences, the latter can give no explanation of the specific effect of the imidazolines. Although, in individual tests, the maximal effect for the specific antagonists is reached sooner than that for the unspecific effective imidazolines, the differences are small, and even the unspecific atropine effect, *e.g.*, in the histamine antagonism, attains the maximum somewhat more quickly.

A further possibility for characterizing the behavior of the reaction consists in the inhibitory effect on systems being investigated about which a specific chemical reaction with the primary stimulatory substances is known, *i.e.*, for example, on enzymes which destroy the latter. It should be stressed that these investigations have not the intention of bringing directly together

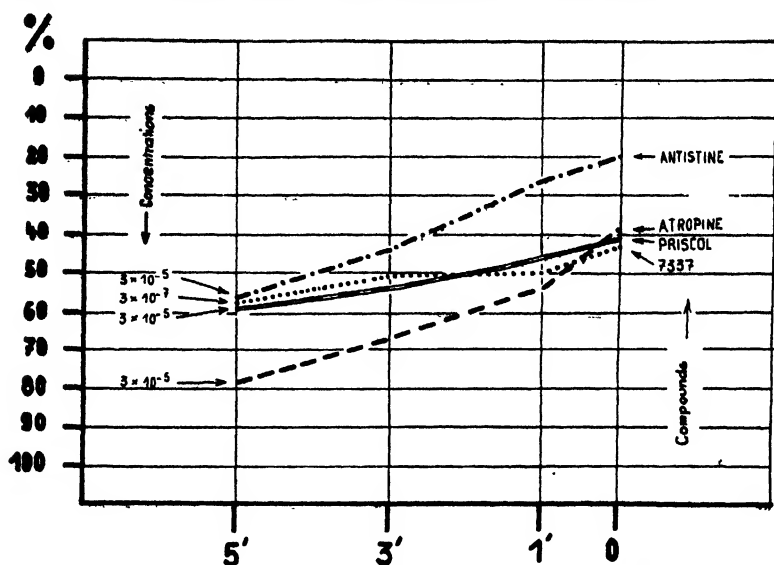


FIGURE 10. Development of antagonistic effect of different drugs to adrenaline with interval between addition of these substances. Abs.—time after application of drug before addition of  $3.2 \times 10^{-6}$  of adrenaline; ord.—inhibitory effect in percentage of response (effect on 5' corresponds to maximal antagonistic effect).

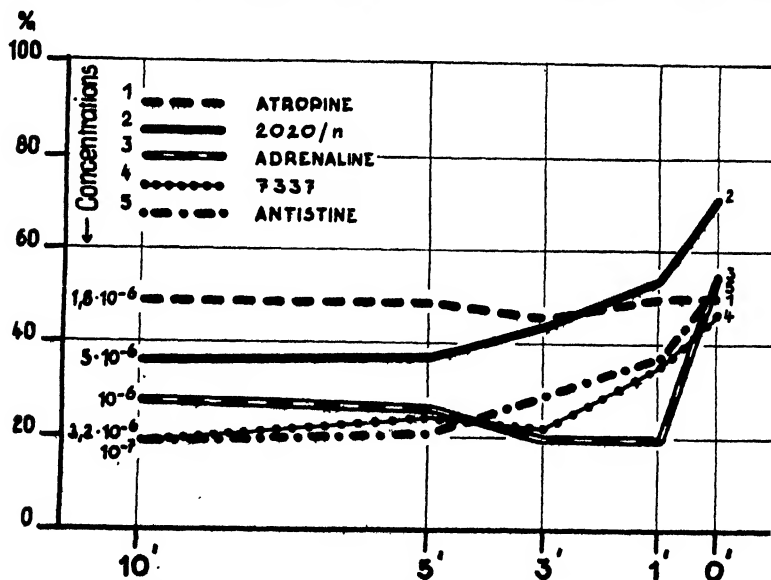


FIGURE 11. Development of antagonistic effect of different drugs to histamine with interval between addition of these substances. Abs.—time after application of drug before addition of  $5 \times 10^{-6}$  of histamine; ord.—inhibitory effect in percentage of response (effect on 10' corresponds to maximal antagonistic effect).

the point of attack with this ferment mechanism, but rather of characterizing the behavior in comparison to the reaction with the specific cell receptors. With this purpose, the inhibitory effect towards diamine oxi-

dase, serum cholinesterase, and adrenaline oxidase was investigated by Schuler in our laboratory. It was found that, in the effect upon diamine oxidase, there exist such small differences between the specific histamine antagonists and other substances that the specific antagonism to histamine

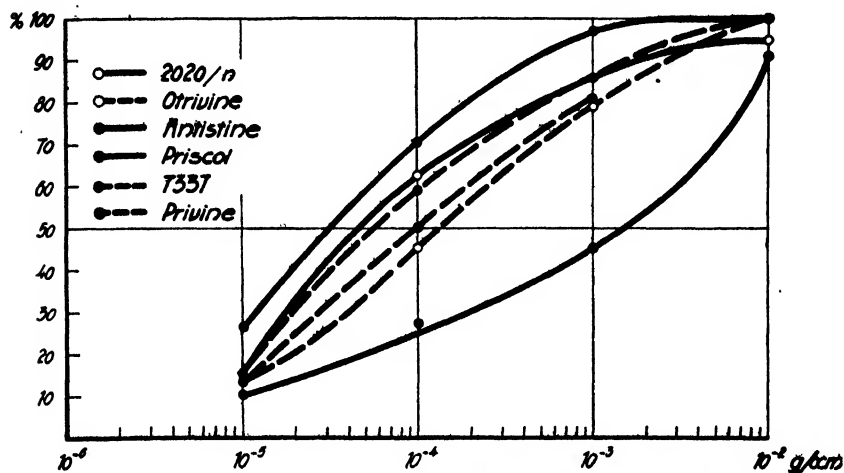


FIGURE 12. Inhibition of the action of diamineoxidase (histaminase), produced by imidazoline derivatives with different specificity of effect: Privine, Otrivine, 2020/n—sympathicomimetic; Prisol, 7337—sympathicolytic; Antistine—antihistaminic. Abs.—concentration of drug; ord.—inhibition in per cent of control after 2 hours.

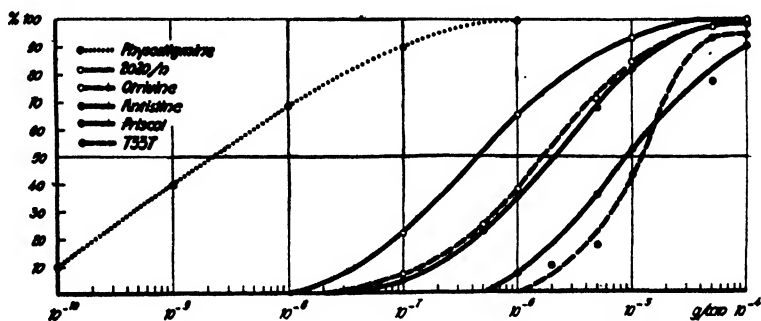


FIGURE 13. Inhibition of the action of serum cholinesterase produced by imidazoline derivatives with different specificity of effect in comparison to Eserine: Otrivine, 2020/n—sympathicomimetic; Prisol, 7337—sympathicolytic; Antistine—antihistaminic. Abs.—concentration of drug; ord.—inhibition in per cent of control after 2 hours.

on the cell substrate must have a different character from the enzymatic reaction (FIGURE 12).

The situation is somewhat different in the case of serum cholinesterase, where greater differences exist. In particular, the effectiveness of some of the sympathicomimetic imidazolines in inhibition of the cholinesterase is relatively pronounced (FIGURE 13). There are in the whole animal, however, no signs of acetylcholine-like effects that could not be in connection with the specific action.



As adrenaline oxidizing agents, a highly active polyphenoloxidase and an amino oxidase have been investigated. The effects on the polyphenoloxidase are uncharacteristic (FIGURES 14 and 15). On the amino oxidase, the sympathicomimetic imidazolines prove the most effective and in the

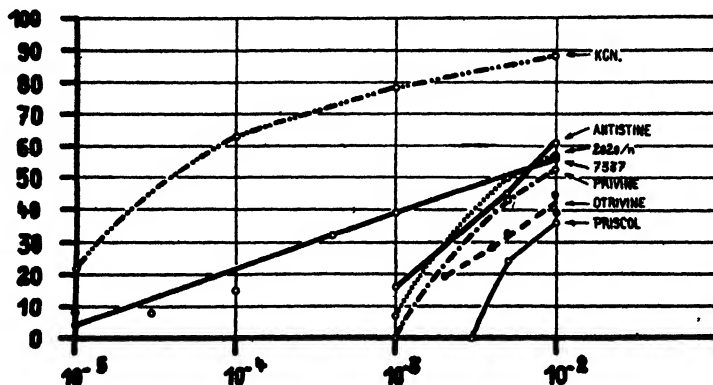


FIGURE 14. Inhibition of the action of Polyphenoloxidase on adrenaline produced by imidazoline derivatives with different specificity of effect in comparison to K.C.N.: Privine, Otrivine, 2020/n—sympathicomimetic; Priscol, 7337—sympathicolytic; Antistine—antihistaminic. Abs.—concentration of drug; ord.—inhibition in per cent of control after 2 hours.

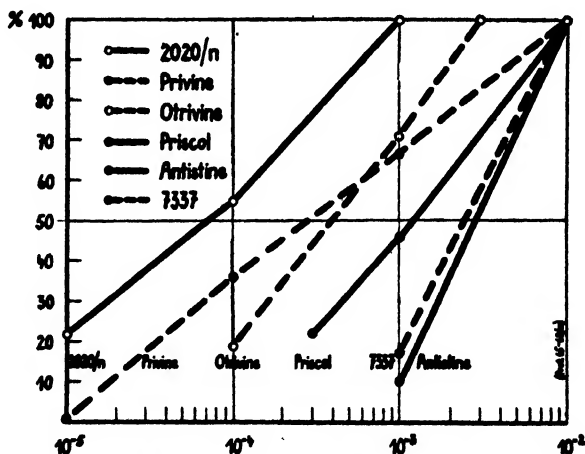


FIGURE 15. Inhibition of the action of adrenalinooxidase, produced by imidazoline derivatives with different specificity of effect: Privine, Otrivine, 2020/n—sympathicomimetic; Priscol, 7337—sympathicolytic; Antistine—antihistaminic. Abs.—concentration of drug; ord.—inhibition in per cent of control after 2 hours.

same order as their relative sympathicomimetic effectiveness in the animal. But the necessary concentrations are much higher for the enzyme inhibition than for the action on pharmacological test objects. On the other hand, the effect of sub-threshold dosages of adrenaline are not reinforced by these products. These results seem only to indicate that there is some similarity in reaction between the aminophenoloxidase and the pharmacological reactive system.

In general, the results of these experiments give no significant possibilities for the explanation of the specific differences, on the basis of general physico-chemical behavior of this group of substances. The expansion of these investigations may change this situation. Also, in the present situation, they may give, just by their negative results, some indication on the behavior of the reactive system.

In the series of the aromatic imidazoline derivatives, substances are found with a chemically similar basic structure, and which possess very distinct specific sympathicolytic, sympathicomimetic, and histaminolytic effects. The specific effects of these substances are differentiated from others belonging to the same pharmacological group, by giving preference to definite points of attack of the specific system. Also, reverse effects are sometimes produced, and effects which belong to other systems can appear.

Consequently, the specific effect shows a system-restricted and an organ-restricted differentiation. This applies to the direct effect as well as to those which only appear as antagonistic effects in the presence of a change in the physiological function, produced by primary effective substances.

The establishment of the reaction equilibrium shown by the dosage-effect curves and time-effect curves and the action on correlated enzymes give no indication (or, only exceptionally, a parallelism) for the explanation of the specific effect.

The exclusion of especially the first-mentioned possibilities gives the basis for the conclusion that a specific reaction of the reactive cell system, independent of those mentioned factors, must be responsible for the specific effect of the substance. The reactive system, independent of what it is, must thus have a high degree of specific differentiated sensitivity towards certain substances of a very similar basic chemical structure and a whole series of others which have more or less different chemical structures. The fundamental difference between the types of specific chemical substances belonging to the same pharmacological group consists in the fact that nearly every group of effective substances has a predilection for only a series of the specific receptors. Thus, the reactive system must be able not only to create the specific effect but also the differentiation of those effects.

This supports the idea that the specific reaction on the cell is caused not so much by the fact that several sharply limited sites of reaction—*e.g.*, sympathicomimetic, parasympathicomimetic, and so on—exist, but that there is in every organ a complex of reactive places which can react with a whole series of chemical compounds in a primary or antagonistic fashion. Only if a substance can react on a definite combination of this reactive complex in a given organ does the specific effect occur. Substances such as adrenaline, histamine, acetylcholine, and others are preferred only because they may react in any organ in the specific way.

This view seems to be in accordance with that of one of the most prominent experts of this field, A. J. Clark,<sup>15</sup> who writes, "It appears to the writer, that the structure of the receptors on which drugs act is probably a subject of similar complexity but that the complexity has been masked by the fact that special attention has been paid to the action of acetylcholine and

adrenaline, which are, however, widespread through the animal kingdom. In these peculiar cases a wide variety of tissues happen to possess receptors of similar patterns with which these drugs can react, and since the pattern is standardized, a fair regularity is observed as regards the action of antagonists; a systematic investigation of other drugs would probably reveal in most cases more erratic actions and antagonism." This, I assume, could be shown by the pharmacological problems discussed.

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## THE MODE OF ACTION OF ANTIHISTAMINIC AGENTS IN THE SKIN

By Charles F. Code, John U. Keating,\* and Milo D. Leavitt, Jr.\*

*Mayo Clinic and Mayo Foundation, Rochester, Minnesota*

Although the skin is the most accessible of our organs, we have considerably less precise physiologic data concerning it than we have concerning such deeply buried organs as the heart or a single neurone of the central nervous system. This is especially true of our knowledge of the function of histamine in the skin.

When the antihistaminic drugs became available, it seemed possible that a study of their action in the skin might offer a means of gaining further information regarding the role of histamine in the skin. This thought, plus the early demonstration of the efficacy of these drugs in certain disease conditions of the skin, led us first to make a study of the antihistaminic action of diphenhydramine hydrochloride (Benadryl) in the skin and then to extend the findings to other compounds of the antihistaminic series. Before these results are presented, however, the knowledge which has accumulated concerning the presence and action of histamine in human skin should be briefly considered.

### *1. The Presence of Histamine in the Skin*

Extracts of human skin have been prepared by Harris,<sup>1</sup> by Pellerat and Murat,<sup>2</sup> and by Nilzén,<sup>3</sup> and after various degrees of purification the histamine content of the extracts has been determined biologically. The amounts of histamine found in the skin by these investigators are closely similar. Harris estimated the amount in normal skin to be about 10 micrograms per gram, with a range of 4 to 24 micrograms per gram for the different layers of the skin. On the basis of determinations in twenty-three subjects, Pellerat and Murat suggested a value for normal skin of about 20 micrograms per gram, with a range of 16 to 24 micrograms per gram. Nilzén tested samples of skin from thirty-three healthy subjects and found values ranging from 5 to 24 micrograms per gram, with a mean of  $10.5 \pm 1.0$   $\sigma = 5.5$ .

The active ingredient in the extracts prepared by these investigators was found by them to be pharmacologically and physiologically indistinguishable from histamine. Histamine, however, has not been isolated in pure crystalline form from skin. Until this is done and the identity of the crystalline product is established chemically, the conclusion that histamine is a normal constituent of skin must remain tentative. Still, it should be mentioned that no evidence has been presented to date which indicates that the H substance in extracts of skin is anything but histamine. The data so far accumulated, therefore, indicate that histamine is a constituent of normal skin and that it is present in skin in physiologically significant quantities.

\* Fellows in Medicine, Mayo Foundation.

## 2. *The Actions of Histamine in the Skin*

If histamine is present in the skin, what is its function there? One approach to the problem that this question poses is to study the action of histamine in the skin.

*A. The Triple Response.* While several workers, notably Eppinger<sup>4</sup> and Sollmann and Pilcher,<sup>5</sup> had reported the effects produced by the introduction of histamine into the skin, Lewis and Grant<sup>6</sup> were the first to study the reaction in detail. They presented what is now the classic description of the action of this compound in human skin, "the triple response." The reaction consists of (1) a localized red spot at the site of injection of histamine; (2) a "flare," or diffuse erythema surrounding the site of injection; and (3) a "wheal" occurring in the region of the injection.

Lewis and Grant demonstrated that the localized red spot is due to intense vasodilatation in the small blood-vessels in contact with the histamine, that the flare is dependent on an axon reflex, and that the wheal arises as a result of increased capillary permeability at the site of injection of histamine. They showed that the response is similar to that given by a whole series of injurious stimuli. Their studies led them to conclude that such stimuli lead to the liberation in the skin of a substance which, in their opinion, is either histamine or a compound very similar to it, and that through it the standard stereotyped triple response is produced. Their work indicates one of the functions which histamine may perform in the skin—that of the common initiator of a defense response to a whole series of noxious stimuli.

*B. Pain.* In the investigations of Lewis and his associates on the skin, injury to the skin, antidromic impulses, pain, itching, and H substance were often closely associated. They, however, never ascribed to histamine or a histamine-like substance the role of chemical mediator of pain stimuli at pain nerve-endings. In fact, Lewis was apparently opposed to the view that histamine may act as an excitant of pain nerve-endings.<sup>7</sup> In so-called "susceptible" skin, some evidence was collected which suggested that a substance other than histamine was involved in the painful and hyperemic state of the skin.<sup>8</sup> Their results, however, led them to conclude that the triple response and the itching occurring after injury were both due to the release of a substance equivalent to histamine.<sup>6,9</sup> Lewis and Marvin<sup>10</sup> also found that antidromic impulses passing down sensory nerves produce vasodilatation by the release of a vasodilator substance. Thus, while Lewis and his associates did not regard histamine or a histamine-like substance as an excitant to pain nerve-endings, the association between H substance and pain-producing structures and phenomena in the skin was often intimate. Also, there seems nothing in their work which actually eliminates the possibility that histamine may be a chemical initiator of pain impulses at pain nerve-endings in the skin.

Rosenthal and Minard<sup>11</sup> have performed some interesting experiments which indicate that a histamine-like substance is liberated, not only when skin is injured, but also when it is stimulated by painful but not injurious stimuli. This substance was liberated from skin shavings of human beings

and animals and from the cornea of rabbits when threshold stimuli were applied. These authors have also demonstrated that very small quantities of histamine produce pain when they are applied to denuded areas of the skin, when they are injected intradermally, or when they are applied topically to the cornea. Lambert and Rosenthal<sup>12,13</sup> have shown that a histamine-like substance may also be liberated from the appropriate cutaneous area when the cervical sympathetic chain or the splanchnic nerves are stimulated.

Ungar and his associates have carried on a series of interesting experiments using gastric secretion as an indicator of the liberation of histamine or a histamine-like substance during various types of stimulation of nerves and skin in dogs. Antidromic stimulation of sensory nerves was found to lead to the production of gastric juice after a latent period of about ten minutes.<sup>14</sup> It was not found possible, however, to identify definitely the substance liberated as histamine.<sup>15,16</sup> While electrical stimulation of the skin and exposure of the skin to cold were without effect, the action of strong irritants and plunging a paw into water at 50° C. for two minutes induced gastric secretion.<sup>17</sup> Such responses were not obtained after degeneration of the nerves of the paw.

In summary, the evidence accumulated to date indicates that histamine or a substance very like it (1) is present in skin, (2) participates in or initiates the triple response, which may be regarded as the response of defense by the skin to noxious stimuli, and finally (3) may be the chemical mediator or initiator of pain impulses at pain nerve-endings in the skin.

### 3. The Effects of Antihistaminic Drugs in the Skin

*A. Effect on the Triple Response.* The first of the synthetic antihistaminic substances, the Fournneau compounds 929, and 1571F, when studied by Staub,<sup>18</sup> were found to be without effect on the triple response to histamine. This finding was later verified by Burchell and Varco.<sup>19</sup> Soon after the introduction of the second group of antihistaminic compounds, the Rhone-Poulenc (RP) series, Parrot<sup>20</sup> showed that the response of the skin to scratching with a needle through drops of 1:100,000 solution of histamine was diminished by the oral administration of 0.2 to 0.4 gm. of Antergan (2339 RP). Vallery-Radot, Mauric, and Halpern<sup>21</sup> have shown in a study of six subjects that Antergan and Neoantergan are both capable of diminishing and in some instances of abolishing the response to histamine in the skin. They gave 0.5 gm. of each of the drugs, 0.4 gm. being administered by mouth and 0.1 gm. being injected intramuscularly.

Elias and McGavack<sup>22</sup> have tested the reaction to histamine in the skin of normal subjects before, during, and after the oral administration of 150 to 300 mg. of Benadryl per day. Histamine was introduced into the skin by means of a pin-prick through a drop of 1 per cent aqueous solution of histamine. Doses of 150 mg. of Benadryl per day definitely reduced both the wheal and flare reactions to histamine. In some cases, after ten days of treatment with 300 mg. of Benadryl per day, the wheal and flare reactions were completely abolished. Using a similar technic, Harley<sup>23</sup>

found that after the oral administration of 300 mg. of Benadryl on one day, followed by 150 mg. a day for six days thereafter, the reactions of the skin to histamine were reduced to a fourth at the end of the first day and to about three-fourths of their original size by the sixth day.

A month or two after Elias and McGavack had reported their results, Friedlaender and Feinberg<sup>24</sup> published observations that they had made on the effect of Benadryl on cutaneous reactions in man. Histamine wheals were induced as a routine by the scratch technic. They found that 100 mg. of Benadryl by mouth did not have a consistent effect on the size of wheals and had no effect on the size of flares induced in response to scratches made through histamine solution. Pretreatment of the area by exposing the scratch site to a 50 per cent solution of the antihistaminic agent for ten minutes did, however, produce a pronounced reduction of the size of both the flare and the wheal. Similar results were obtained with flares and wheals produced in sensitive patients by ragweed antigen. Preliminary studies with tripeleennamine hydrochloride (Pyribenzamine) indicated that it was at least as effective as Benadryl in reducing the whealing reactions. Arbesman, Koepf, and Miller<sup>25</sup> found that the oral administration of Pyribenzamine in doses of 50 to 150 mg. decreased the size of histamine skin wheals in eighteen of twenty-eight subjects.

Cohen and associates<sup>26</sup> administered histamine to the skin in various concentrations by iontophoresis. When 50 mg. of Benadryl were given orally four times a day for one to seven days, solutions of histamine which were two to thirty-two times as strong as those used in control tests were required to produce a whealing reaction in the skin. Benadryl given by iontophoresis also raised the cutaneous threshold to histamine. Aaron and Abramson<sup>27</sup> observed that areas of skin treated with 5 and 10 per cent solutions of Pyribenzamine by iontophoresis lost or nearly lost the whealing response produced in normal skin by the iontophoretic administration of 1:10,000 and 1:100,000 dilutions of histamine phosphate. While the first two synthetic antihistaminic compounds studied, 929 F and 1571 F, are apparently without action on the triple response, the investigations summarized in previous paragraphs clearly show that the more recent members of this group of compounds, Antergan, Neoantergan, Benadryl, and Pyribenzamine, do exert an effect on the action of histamine in the skin. They all reduce the reactivity of the skin to histamine. With the exception of the work of Cohen and associates, the results allowing this conclusion, while they have demonstrated the points of interest to the authors, have, for the most part, been qualitative and have, in the main, been based on observations of the whealing response to histamine. There are few or no definitive data in the literature dealing with the effect of the antihistaminic compounds on the flare component of the response to histamine.

*B. Action on Pain Nerves.* Rosenthal and Minard<sup>11</sup> were the first to demonstrate the anesthetic action of antihistaminic drugs. They noted that thymoxyethyl-diethylamine (929 F), when injected intracutaneously or applied locally to denuded skin in 1:200 dilution, produced local anesthesia similar to that obtained with 1:100 solution of procaine hydrochloride but of

longer duration. When given orally or rectally in sufficient amounts to dogs, monkeys, and guinea pigs, the drug generally abolished the pain responses to pinching, pricking, and cutting and raised the electrical threshold of the skin, without, however, affecting that of somatic, sensory nerve trunks. Rosenthal, Minard, and Lambert<sup>28</sup> have confirmed these findings and have noted a similar reduction of the threshold of abdominal visceral sensation after administration of the drug.

Burchell and Varco<sup>19</sup> observed some general lowering of the pain threshold in dogs receiving large doses of thymoxyethyl-diethylamine (929 F) subcutaneously. In human skin, anesthesia occurred over blebs produced by the intracutaneous injection of 929 F and 1571 F, while none occurred when the drugs were given subcutaneously.

Very few tests for anesthetic properties have been made with the newer antihistaminic compounds. The French pharmacologists apparently have not been much interested in this potentiality of the compounds. Halpern,<sup>29</sup> in his extensive report on Antergan, simply asserted that compounds of the series 2325 RP have significant local anesthetic activity, but he did not give data in support of the statement and made no further comment regarding it. Clinical investigators in France have suggested and observed on a number of occasions that the administration of antihistaminic compounds will produce a reduction of the pain of shingles and certain causalgias.<sup>30,31</sup> Dews and Graham,<sup>32</sup> in England, reported that Neoantergan, when given intracutaneously to guinea pigs, produced local anesthesia and that its local anesthetic potency was approximately three times as great as that of procaine. They also noted some mild though variable analgesia in rats receiving the drug.

#### 4. Summary of Experimental Data

Because of the possible role of histamine at pain nerve-endings in the skin, it was decided to investigate further the anesthetic action of the antihistaminic compounds. Anesthetic activity of these compounds was first tested. Then experiments were done to determine whether or not there was a relationship between anesthetic action and antihistaminic effects.

Benadryl (diphenhydramine hydrochloride) was used in the first series of experiments. The results obtained with this compound were then extended in a second study, in which a comparison was made of the actions of Benadryl,\* Pyribenzamine\* (tripelennamine hydrochloride), Neoantergan\* (ethylenediamine, N-p-methoxy-benzyl-N', N'-dimethyl-N-2-pyridylmoleate), and the two compounds derived from thiodiphenylamine,\* beta-dimethyl-aminoethyl-N-thiodiphenylamine (3015) and beta-dimethylamino-alpha-methyl-N-thiodiphenylamine (3277) of Rhone-Poulenc.

It was first observed that when Benadryl was injected into the skin local anesthesia was produced at the site of the injection. Benadryl, in dilutions of 1:500, 1:1,000, 1:5,000, 1:10,000, and 1:20,000, was found to possess anesthetic effects in the skin equal to procaine in dilutions of 1:200, 1:400, 1:800, 1:1,600, and 1:3,200, respectively.<sup>33</sup>

\* The drugs used in this study were kindly provided by the manufacturers: Benadryl by Parke, Davis & Company; Pyribenzamine by Ciba Pharmaceutical Products, Inc.; and Neoantergan and the thiodiphenylamine derivatives by Société Parisienne d'Expansion Chimique Spécial.



The effect of the antihistaminic drugs on the size of flares resulting from the intracutaneous injection of histamine was used as a means of quantitative assay of antihistaminic action. The flares were produced under standard conditions and the areas were measured from photographic records.

In the first study, when the action of Benadryl only was being tested, control flares were always made on the same occasion as the test flares. In the more complex series of studies, in which the five antihistaminic agents were compared, space did not permit simultaneous inclusion of the control flares and, for this reason, they were made on other occasions. An extensive series of observations was made to determine the variability in the size of control flares made simultaneously and at different intervals of time to cover the experimental circumstances. The results obtained led to the development of standard assay procedures applicable to the experimental conditions.

The reduction in the size of histamine flares following the oral, intravenous, and intradermal administration of Benadryl was first confirmed. Experiments designed to determine the degree of dependence of the flare-reducing effect of Benadryl on its anesthetic action in the skin were then carried out. By the use of the assay procedures developed during the study, the flare-reducing properties of a standard local anesthetic agent (procaine hydrochloride) were tested. The observation first made by Lewis and Grant,<sup>6</sup> that cutaneous anesthesia induced by procaine reduces or eliminates histamine flares, was confirmed. The possibility arose that the antihistaminic action of Benadryl injected into the skin is dependent on the anesthesia it produces. If this were the case, then equianesthetic doses of Benadryl and procaine should have similar flare-reducing effects. When tests were made, however, it was found that Benadryl consistently produced a more pronounced reduction in the size of the flares than procaine of equianesthetic strength. Some separation between the anesthetizing action and the flare-reducing action of the drug was indicated.

By the use of similar doses of procaine and Benadryl, tests were then made of their effects on size of flare after the anesthesia that they produced in the skin had subsided. It was found that even when the anesthetic action of Benadryl in the skin had disappeared, its flare-reducing effects persisted and indeed the flare-reducing action of the drug was as potent in the absence as in the presence of the anesthesia. On the other hand, the flare-reducing action of procaine, after its anesthetic effects had disappeared, was slight or absent. The effect of Benadryl on histamine flares was not then directly dependent on the local anesthesia that it produced in the skin.

In the second study, the flare-reducing potencies of the five drugs (Benadryl, Pyribenzamine, Neoantergan, 3015, and 3277) were first compared. When similar molar concentrations of the drugs were used, it was found that Pyribenzamine and Neoantergan had the greatest effect on the size of the flares, while Benadryl was intermediate between these two drugs and the two thiodiphenylamine derivatives, 3015 and 3277, which had the least and approximately equal effects on size of flare.

The anesthetic actions of the drugs were then compared. It was found

that they all produced local anesthesia in the skin when injected intradermally. Similar molar concentrations of the drugs were used, and the duration of anesthetic effect which they produced was tested. Based on this measure of anesthetic potency, 3277 and 3015 had nearly equal and, at the same time, the most potent action of the series of drugs. There were only minor differences between Neoantergan, Benadryl, and Pyribenzamine, but they were all distinctly less effective than the thiodiphenylamine derivatives. Since the order of potency was the exact reverse of that noted for the flare-reducing action of the drugs, the conclusion was drawn that the effect of the drugs on size of flare is not directly dependent on their anesthetizing action in the skin.

As a final step in the study, the flare-reducing actions of the compounds were tested one hour after their injection into the skin, when the anesthesia that they had produced had subsided. Even in the absence of anesthesia, the drugs continued to show considerable flare-reducing activity.

The experiments allow the conclusion that the flare-reducing action of the antihistaminic agents studied is not directly dependent on the presence of the local anesthesia which they produce in the skin. Lewis and co-workers have shown that the flare produced by the injection of histamine is due to an axon reflex. It seems most probable that the flare-reducing action of the antihistaminic compounds is due to some effect on nervous tissue in the skin. A direct action on the blood-vessels might be involved; but this seems unlikely, for the antihistaminic drugs could hardly have diffused a sufficient distance from the site of their injection to reach all the minute blood-vessels ordinarily involved in a histamine flare. Also, if a direct effect on blood-vessels were the cause, one would expect to see, at times, no flare in the immediate vicinity of the site of injection of the antihistaminic drug and flaring at some distance from the injection. This was never observed. Some action on nerve tissue, therefore, seems the most likely explanation of the drug effects.

The separation between antihistaminic and anesthetic actions may then be a result of differences in degree of action on nerve tissue rather than a separate action on different tissues. Following this argument, it may be concluded that the impulses which are normally generated by the injection of histamine and are carried antidromically to the blood-vessels in the flare region are apparently interrupted or are not initiated when an antihistaminic compound is present. This dislocation persists even after sensitivity to pain, as tested by electrical stimulation, has returned to the skin. The mechanism of initiation of the impulses giving rise to the flare may be closer to the minimal disturbance which will excite the nerve channels involved than the disturbance induced by electrical stimulation of the skin. Under such circumstances, concentrations of the antihistaminic compounds which produce no effect on pain-impulses might, nevertheless, block flare-producing impulses. Such an effect offers an explanation of the anti-flare action of the drugs following their oral and intravenous administration, when no anesthesia in the skin is present. It also serves to explain the persistence of antifiare action after a subsidence of anesthesia following

the intracutaneous injection of the drugs. Further experimentation will be required, however, to determine whether this is the mechanism of action of these drugs and whether their action on nerve-tissue is directed toward nerve-endings or toward nerve-trunks in the skin. The elucidation of such points as these will undoubtedly lead to a clearer understanding of some of the effects of histamine in the skin.

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# ANTIHISTAMINE THERAPY. EXPERIMENTAL AND CLINICAL CORRELATION

By Samuel M. Feinberg

*Division of Allergy, Department of Internal Medicine, Northwestern University Medical School, Chicago, Illinois*

In a discussion or evaluation of an antihistaminic drug from the clinical standpoint, we should ask ourselves three major questions: (1) What manifestations are helped, to what extent, and what are the limitations of its use? (2) What are the nature, extent, and significance of the undesirable actions of the drug, and how can they be remedied? (3) What are methods by which such a drug can be evaluated prior to clinical experience with it? In other words, what experimental methods will give us the best correlation with clinical results?

The purpose of this paper is to present the highlights of the facts pertaining to each of these questions. The clinical and experimental findings which I shall discuss are primarily those encompassed in my own experience in my practice and laboratory. For the most part, the points elaborated here will concern themselves with the antihistaminic drugs as a group, rather than with the individual drugs. Because experience with many of these drugs is in its initial stages, I shall refrain from making any sharp clinical or experimental comparisons, which could be easily modified by future more extensive observations.

## *I. Clinical Results*

*Seasonal Hay Fever.* With most drugs, about 60 to 80 per cent of the seasonal hay-fever patients obtain some degree of improvement in their symptoms.<sup>1,2,3</sup> The degree of relief varies from slight to excellent, but rarely is it complete. It is exceedingly important to realize that the incidence and degree of relief are determined by many variables besides the potency of the drug. One of the most variable is the individual patient. Irrespective of the severity of the symptoms, some patients do not respond favorably to the antihistaminic drugs. What is even more difficult to understand is that this individuality may extend to a selective response to some members of the antihistamine group while, to others, the response is unfavorable. In another person, this selective response may be reversed. It is this peculiar behavior, together with individual differences in the production of undesirable symptoms, which makes it of advantage to possess a range of antihistaminic drugs from which to select the most desirable one for the particular patient. As a corollary, it is also this characteristic which should encourage the production of a reasonable number of new antihistaminic agents, even though their incidence of effectiveness may be no greater than, or as great as, previous products.

In evaluating any antihistaminic drug in hay fever, one must bear in mind that a number of conditions determine its action. Some of the hay-fever symptoms, such as sneezing and rhinorrhea, are readily helped, while

others, such as nasal blocking and headache from edema of sinus mucosa, may be resistant. The type and variety of hay fever make considerable difference. Grass-pollen cases will respond more easily than ragweed cases, and mild ragweed cases will do better than severe ones. The severity of the particular season has an influence. For example, some ragweed-sensitive patients who responded well in the 1946 ragweed season in the Central States did not do so well in 1947, for no other reason than that the latter season was more intense.

Geographical differences in pollen prevalence have a profound bearing on the response of hay fever to antihistaminic drugs or to desensitization therapy. It is one thing to combat symptoms in a locality which has a pollen count of 50 at the height of the season and another to meet conditions in a locality like Chicago, where the count at the height of the season is 800 or more and a count of 50 generally occurs before August 15, the date which the average layman has accepted as the official opening date for the season.

It is also a common experience that the antihistaminic drugs which have been so helpful at the beginning and height of the hay-fever season may be totally ineffective during the later stages, when the swelling of the mucosa is apparently less labile and when weather factors play a predominant role. From all these facts, it is easy to see why patients who have had their hay fever mitigated by desensitization therapy respond much better to antihistaminic drugs than do the undesensitized patients.

Other conditions having to do with physiological states determine the efficacy of these drugs. A dose of the drug taken on an empty stomach may be markedly effective, whereas the same dose taken after meals may fail to work. There are other less definable body states which apparently have an influence in causing variation in effectiveness of these drugs from time to time in the individual patient.

*Perennial Vasomotor Rhinitis.* The incidence of relief in chronic vasomotor rhinitis is from about 30 to 60 per cent with most drugs, depending on the drug and the nature of the cases encountered. The question arises: Why is the incidence of relief less than in the seasonal cases? One or two possible explanations are offered. Most patients with perennial rhinitis present the symptoms of persistent nasal blocking as the major complaint. This complaint resembles the condition that prevails in some patients at the end of the hay-fever season, where we have also observed that the effectiveness of these drugs is diminished. To substantiate this view further, we have noted that very early cases of perennial vasomotor rhinitis, whose major symptom is sneezing, respond as well as the seasonal cases. Another possible explanation is that in perennial rhinitis we may be dealing with an appreciable percentage of instances of intrinsic causes which may perhaps not respond in the same manner as extrinsic causes.

*Asthma.* The typical asthma attack is influenced very little by the oral use of the present antihistaminic drugs. This fact is so generally overlooked and so commonly misunderstood that one cannot overemphasize it. In the past few years, I have had not a week go by without observing one or several new patients who had been having persistent asthma without

relief and whose major medication had been Benadryl or Pyribenzamine. Frequently, a few doses of the older remedies—ephedrine, aminophylline, epinephrine, or iodides—were sufficient to break up the attack. On the other hand, the addition of an antihistaminic drug to the other therapy may make for more effective relief in selected instances of asthma. The spasmodic cough without dyspnea or wheezing, which is a type of bronchial allergy frequently seen in children, responds favorably to these drugs in 25 to 60 per cent of the cases.

Two likely explanations suggest themselves for the lack of response of asthma to these drugs. It may be that in asthma we are dealing with a mechanism different from, or additional to, histamine. Another possibility is that the local release of histamine in the bronchi is of a magnitude greater than can be combated by the amount of antihistaminic drug which can be tolerated. It is possible that new drugs with much less toxicity or greater potency may permit the solution of this problem. It is also possible that the topical application of the antihistaminic drug by aerosols might solve this situation. We have experimented clinically with aerosols and have had some encouraging but inconstant results. The obstacle may lie in the difficulty of getting a sufficient amount of the drug to the bronchi by the ordinary simple methods. We have also experimented with micronized drug inhalations, which offer some interesting possibilities. Meanwhile, we have turned to extensive experimentation, in guinea pigs, with aerosols of the drugs in their ability to prevent bronchospasm induced by histamine aerosols. We have studied the concentrations required and the effectiveness, tolerance, and duration of action of a large series of antihistaminic drugs. The detailed findings will be presented elsewhere.<sup>4</sup> Here I wish merely to call attention to the following facts: aerosols of these drugs are effective in the prevention of bronchospasm from histamine aerosols; the duration of action is short, averaging about an hour; and some drugs are very irritating when administered in this manner.

*Urticarial Dermatoses.* Urticaria and angioneurotic edema respond well to these drugs, producing improvement in 60 to 80 per cent of the cases.<sup>2,5,6</sup> The itching is the symptom most consistently helped, while the edema may be improved moderately or not at all. In severe and dangerous angioneurotic edema, such as edema of the larynx, more prompt results can be expected from epinephrine hypodermically. It is best to give the latter and the antihistamine drug at the same time. The delayed serum-sickness type of reaction frequently encountered a number of days after the administration of serum, penicillin, or sulfonamides also responds well to these drugs. The joint symptoms, however, are more resistant. In all such conditions, the benefit is only temporary, lasting from three to eight hours with each dose. The syndrome pursues its usual course and duration. Dermographia is also benefited by such drugs, and the moderate dermographia, so frequently an obstacle to the interpretation of diagnostic skin tests in allergy, usually can be controlled by the prior administration of a dose or two of the drug.<sup>6</sup>

*Allergic Dermatitis and Other Dermatoses.* The itching of atopic dermatitis (flexural eczema) is helped in 50 to 80 per cent of the cases.<sup>3</sup> In some

instances, the condition of the dermatitis may even improve, because of the discontinuance of scratching and the correction of excessive histamine damage. Some patients require medication only at night, while others gain more by the use of the drug several times daily. In many instances, the use of an ointment of the drug is of additional advantage.<sup>7</sup> Contact dermatitis responds also, but not as frequently, to the use of these measures. Pruritus ani and vulvae are frequently ameliorated by these drugs, and Pyribenzamine ointment has been found to be an excellent palliative remedy in pruritus ani. Many instances of other types of pruritus, as well as insect bites and dermatophytosis, also respond to the drug orally.

*Miscellaneous Manifestations.* Migraine headaches are only occasionally relieved. Gastrointestinal allergy is improved in some instances, although this action is often handicapped by the tendency of these drugs to produce gastrointestinal upsets in some patients. These drugs are helpful in the prevention of systemic reactions from antigen administration, but their help should be regarded only as moderate, since they do not prevent such reactions from large increments of the antigen. In the treatment of systemic reactions, epinephrine, ephedrine, and aminophylline are much more trustworthy, but the antihistaminic drugs may be of additional aid. In histamine reactions from cold, heat, and alcohol these drugs have been found of help. The erythema, itching, and some of the edema of dermatomyositis have improved with the use of these agents.

*Other Factors in Treatment.* As in hay fever, the relief obtained with the antihistamine drugs in the conditions listed is seldom complete and depends on many variants, some known and some unknown. The usual duration of action from a single oral dose is three to eight hours, although in some instances it may be longer. The different drugs vary some in this respect, and it is suspected that one or two in particular may produce unusually long relief. However, this will require more extended and concise studies. I am satisfied that the vast majority of reports of duration of action given by patients cannot be trusted. Many patients will report that a dose of the drug has relieved their hay fever for twenty-four hours. Actually, the condition is as follows: The patient has hay fever in the morning. Without the use of any medication it might last only a half-hour to two hours, not to recur until the following morning. If the antihistaminic drug is administered and the symptoms disappear in about an hour, not only is it impossible to determine the duration of action, but it is even dubious whether the drug has produced any relief. The dose administered, the potency of the drug, the individual responsiveness of the patient to the drug, and the severity of the symptoms are factors which influence the duration of action.

Increasing experience with these drugs indicates that their continued use tends to diminish the effectiveness of therapeutic response in many instances. Whether this is due to a true tolerance or to psychic factors is not certain. It is possible that, once the novelty of a partial relief has worn off, the patient centers his attention on the unrelieved portion of his symptoms.



*Dosage and Administration.* Of the drugs studied by us thus far the effective average adult dose of most of them has been 50 mg. With some drugs, it has been possible to obtain results at times with 12.5 to 25 mg., while, with other drugs, 100 mg. doses were usually required. A number of additional factors determine the dose. Among these are the severity of the symptoms, whether the medication is taken on a full or empty stomach, and the limitations due to its toxic effects. There are, no doubt, additional unknown factors which determine the effective dose. Children tolerate proportionately high doses. Children over 10 usually tolerate adult doses, and sometimes better than adults. Younger children usually respond to 50 per cent of the adult dose, while infants as a rule require 20 to 40 per cent of the adult dose. In most instances, the drug should be administered as needed, rather than given at stated regular intervals. This will obviate unnecessary medication and will reflect more accurately the effectiveness of the drug. There is no excuse for administering the drug several times daily for an allergic attack which recurs only periodically.

Some physical and chemical considerations are of practical importance. The pill is the common form of such medication; yet, because of the bitter taste of most of these drugs, the pill becomes objectionable, unless it is sugar-coated. Some of the pills are so soft and unpleasant that the patient prefers other drugs which may be less potent but which come protected in capsule or by a pleasant coating. Liquids, usually elixirs, are also handy forms of medication. The amount of drug in one unit (capsule or tablet) has a definite bearing on the success of the therapy. If, for example, the usual effective dose of a drug is 50 mg. and it is dispensed in 25 mg. capsules or pills, a certain number of the patients will fail to take the prescribed 50 mg. because they will suspect it to be a double dose, even though they have been advised to the contrary previously. Thus, such patients may contribute to a reduction in the figures in the evaluation of the drug.

Hypodermic injections have been used, particularly of Benadryl, Antistine, and Pyribenzamine. They are seldom indicated but may be of use in emergencies. They should be employed with great caution, since their toxic action is not yet fully appreciated or understood. Aerosols may be of help in some cases of asthma. Topical applications in the nose and eyes may ultimately be of some aid, but in most instances and with most drugs the primary irritation is very objectionable. Applications on the skin in the form of ointments have been found valuable by us.<sup>7</sup> This came as an outgrowth of our experience with the inhibition of the dermatographic wheal, when a drop of a solution of an antihistaminic drug was applied previously to the intact skin. It is possible that other forms of application of these drugs may also be of advantage.

## II. Side Actions

The usefulness of the antihistaminic drugs is considerably hampered by a number of undesirable effects.<sup>1,8</sup> These untoward actions, although differing in degree in different drugs, are, for the most part, similar to all and present a characteristic pattern.

*Sedation.* The most consistent side action of all antihistamine drugs is sedation, or sleepiness. Indeed, it is so common an attribute and so constantly present, if the size of the dose is large enough, that it may be considered as much a pharmacologic action of these drugs as are their antihistaminic properties. Some patients have discovered that they can substitute these drugs for barbiturates when they desire more complete hypnotic effects. This sedative action appears to be peculiar to man, and it is interesting to speculate on its possible significance. Among the possible explanations for this phenomenon, the following have been considered: (1) that histamine is normally the mediator of sensory functions and that displacement of histamine results in sedation and analgesia; and (2) that this has nothing to do with antihistamine functions. The degree of sedation may vary from a slight diminution in alertness to deep stupor. The drowsiness, grogginess, and diminished alertness may not only be disturbing to mental concentration but may diminish actual physical energy. Furthermore, the mental foginess can result in serious hazards and may lead to accidents.<sup>8,9,10</sup> The intensity of the sedation is a difficult thing to assay, particularly in the milder forms, because of the individual interpretations. Although, after considerable trial of a drug, we can obtain an idea of its sedative index, an enormous amount of clinical material and time are needed for this evaluation. An objective method of determining the effect of a drug on sedation or alertness would be more satisfactory, and we are now planning such a procedure.

Other symptoms occur which may be variations of the sedative action. Lassitude, diminished alertness, and diminished powers of concentration are undoubtedly part of the same effect. These symptoms are not to be ignored, since they may have a profound effect on the quality and quantity of physical and mental output. Disturbed muscular coordination and aching of muscles, particularly of the eyes, are probably related symptoms. On several occasions, I have observed temporary sexual impotence produced by antihistamine drugs. It may even be that the irritability, nervousness, and palpitation are, in some instances, due to the attempt to overcome the sedative action.

*Factors in Sedation.* A number of factors contrive to determine the occurrence and degree of sedation. The individual drugs differ. For example, Benadryl produces sedation in 50 per cent, Pyribenzamine in 25 per cent, and such drugs as Antistine and NH-188 are remarkably free from sedation. Some persons are more prone to this effect than others. The sedative action is more pronounced in adults than in children. It is remarkable how often it happens that, when a mother and her youngster of 10 or 12 take the same dose of the drug, sleepiness results for the mother and not for the child.

The individual response to different drugs varies. One drug will cause sedation in A and not in B, while another drug may produce the reverse effects. A number of other conditions determine sedative action. Taking the drug after meals may produce no toxic symptoms, while the same dose on an empty stomach may result in profound sedation. Conditions of

weather, state of fatigue, degree of physical activity, and use of coffee or tea may affect the sedative action. It is common experience that, after the first few doses, a tolerance to the sedative effect may be acquired. No doubt there are other factors as yet unknown which have a bearing on this action.

Can the sedative action of an antihistaminic drug be appraised by acute toxicity experiments on animals? The obvious answer from the known pharmacologic data is "No." Clinical assay of sedation presents a number of difficulties. If the observer fails to question the patients, many instances of sedation are missed. If he does question them, the objection may be raised that the suggestive influence may be misleading. Many patients are very poor observers and fail to note anything unusual even if they stumble about in a fog. On the other hand, many individuals are very prone to misinterpret symptoms. Objective tests of the drugs on human alertness will have to be developed. Another possibility is the determination of sedative effects in monkeys or apes.

*Correction of Sedation.* Frequently, difficulties with sedative effects can be corrected by substitution of other antihistaminic drugs. Where this is not possible, the addition of stimulants may solve the problem. Racemic and dextro-amphetamine, desoxyephedrine, ephedrine, aminophylline, coffee, and Benzadrine inhalers constitute a choice of approaches. But this balancing of two opposing effects is far from a simple problem. No type or amount of stimulation fits all stages of sedation encountered. Furthermore, if the stimulant action is too great, a new train of undesirable symptoms ensues. Then there is the very delicate question of balancing the duration of action of the two opposing drugs, which seldom seem to neutralize each other completely for the entire period. It is not at all surprising, therefore, that we regard this problem as being far from settled. We believe the difficulty will not be solved until the molecular structure of the antihistaminic drug is properly modified to remove this undesirable activity, providing the sedative action is due to the drug itself.

*Other Toxic Effects.* Dizziness is a common symptom. Dryness of the mouth, throat, and nose is a frequent symptom and, if not too marked, may be of advantage, particularly in allergic rhinitis. Sometimes, however, it reaches undesirable proportions. Nervousness, excitability, insomnia, and palpitation are complaints of some persons, although oddly enough the same people will at times complain of sleepiness from the same drug. Disturbance of appetite is a common effect and gastric discomfort is not infrequent. At times, abdominal pain and diarrhea may result. Three or four instances of convulsive seizures have been described. It should be noted that, thus far, all drugs examined by us are local anesthetics. Although one may speculate on the possible relationship of the anesthetic and antihistaminic functions,<sup>11</sup> quantitative and qualitative experiments do not support the view that the one type of function is dependent upon the other.<sup>12</sup>

In some patients, we have observed marked burning in the epigastric and esophageal regions, particularly with one drug. Burning on urination has been noted occasionally. A few instances of dermatitis<sup>13</sup> have been encountered. More serious and delayed toxic effects, perhaps hematopoietic,<sup>14</sup>

hepatic, or neurologic, may ensue as observations continue. Thus far, the reported serious effects are meager, but it is not amiss to emphasize that these are potent drugs whose pharmacology, toxicity, and mode of action are not entirely understood. A cautious observant attitude with any of the new drugs may save us from calamities.

### *III. Methods of Evaluation*

An important step in the launching of a new drug is its assay or evaluation prior to release to the general medical profession. Let us examine the methods available for the antihistaminic drugs in order to see which may be of significance and which may have the greatest bearing on the clinical problem.

*Laboratory Methods.* Many laboratory methods have been proposed, but I shall briefly discuss only a few of them. The most common method concerns the protection conferred by the injection, usually intraperitoneally, of the drug in the guinea pig, to be followed by lethal doses of histamine intravenously. The first type of this technique aimed to find out the maximum number of lethal doses of histamine the animals will tolerate after the injection of a rather large amount of the drug. On that basis, a drug which protects against 50 times more histamine than another certainly does not possess such a relative clinical potency. We soon realized that such amounts of histamine are entirely too far removed from physiologic consideration in allergy and even in anaphylaxis. At the suggestion of Dr. Dragstedt, we modified this technique to observe the smallest amount of the drug which would protect the animal against 1 MLD<sub>100</sub> of histamine, since that comes much closer to probable conditions of histamine liberation in anaphylaxis.

Another measure of antihistamine action concerns the prevention of bronchospasm from aerosolized histamine<sup>15</sup> after prior administration of the drug. The advantage here is that the animal does not have to receive a fatal dose of histamine and that the degree of histamine intoxication and symptoms more closely approach those of clinical allergy. We have further modified this technique by using aerosols of the antihistaminic drugs as protective agents.

The effect of the drug on the histamine contraction of the guinea-pig ileum is a favorite method of assay for antihistamine potency. In going over the literature and unpublished reports of various experimenters with this technique, one is struck by the fact that practically everyone has a different modification. One aims for total inhibition, another for 50 per cent, and still another for 75 per cent. The amount of histamine, the size of the strip, the timing, and many other factors vary. It is not surprising that one cannot safely compare the findings of one experimenter against those of another. Even when a standard technique is followed in one laboratory, such as ours, it is noted that innumerable and imponderable difficulties beset this procedure. It is a sensitive method, but, at best, it measures only the action of a drug on a piece of muscle, removed from living conditions and derived from an animal which is far removed from man. The inhibition of the depressor effect of histamine on the dog's or cat's

blood pressure is another favorite method of study, but here again it is doubtful whether this is a measure of the drug's clinical worth.

Since antihistamine action is presumably not the only attribute of the drug desired, it is but natural that experimenters have turned to inhibition of anaphylaxis<sup>16,17,18</sup> as an indicator of the antiallergic properties of these drugs. This is probably as good a laboratory method as any. There are some objections to this technique, nevertheless, when one begins to make extensive comparative studies. The variability of anaphylaxis would mean that for conclusive work in all phases one would have to sacrifice tremendous numbers of animals. It has the further objection that in intensity of action it gets a considerable distance away from conditions which must prevail in connection with clinical allergy. The recent claim that antihistaminic drugs have an inhibiting effect on experimental lesions of serum sickness and periarteritis nodosa in the rabbit deserves further investigation.

One might expect that anaphylaxis *in vitro* would solve these problems. However, we have found such marked variability in sensitivity as to make many of the quantitative tests invalid. Since most of the allergic reactions concern themselves with vascular changes resembling whealing, it was thought that assays of these drugs on histamine wheals in rabbits would furnish a more accurate means of assay.<sup>19</sup> The whealing in rabbit skin is of a poor order and difficult to measure, however, and in my opinion it is not a good method.

*Whealing in Man.* Since increased capillary permeability in man is the most common characteristic of allergy, I have thought that studies of the effect of these drugs on histamine and other wheals on the human skin might give us a more accurate reflection of the activity of a particular drug. Long ago, we had shown that a solution of an antihistaminic drug applied to a scratch a few minutes prior to the application of a histamine solution inhibited or diminished the wheal from the latter.<sup>20</sup>

It is worthy of note that the whealing from other causes, such as antigens, codeine, atropine, eserine, *etc.*, were similarly inhibited. The procedure for assay by this method in its simplest forms is as follows: Several rows of scratches about one-quarter inch in length are made on the back of a subject. A drop of a standard solution of the drug to be tested is placed on each of six scratches in one row. On other rows, different antihistaminic drugs are placed for comparison. At the end of about 10 minutes the solutions are washed off. Each row of scratches is then treated with serial dilutions of histamine (usually from 1:1,000 to 1:32,000 histamine in terms of base). One row is treated with histamine without previous pretreatment with the antihistaminic drug. The height and degree of inhibition give us an idea of the activity of the drug.

We believe this method and modifications of it offer probably a closer approximation than any others of the anticipated behavior of the drug in allergy. We are utilizing this method not only in comparative evaluations of the antihistamine effects of various drugs, but also to study the quantitative relationship between histamine and antigen reaction in order to procure information which might throw light on the mechanism of the

allergic reaction and on the mechanism of action of these drugs.<sup>21</sup> At this time, we shall not attempt to give details of our findings, except to say that by this means we have been able to demonstrate decisive and consistent differences between some of the antihistaminic drugs.

*Clinical Assay.* The final worth of a therapeutic product is, of course, judged by its behavior in a thorough clinical trial. I would like, however, to enumerate briefly some of the pitfalls and difficulties attending evaluation by clinical means. All the variables which we discussed previously as affecting the therapeutic results from the antihistaminic drugs in hay fever operate to modify the clinical assay of such drugs. These include the severity of the disease, its stage, the type of hay fever, the severity of the particular season, the geographical location of the trial, and whether the patients are those who have partial immunity from desensitization or those who have been entirely untreated. The size of the dose used reflects on the general results, and even the amount of drug in one tablet or capsule has influence on the total amount of drug the patient is apt to take. The reliability of the patient is of great importance, and frequently in suspicious cases we have been able, by one procedure or another, to decide that the patient's report is not to be trusted.

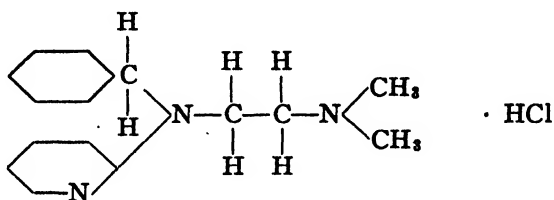
Still more difficult to judge is the duration of action of these drugs clinically. Only now and then can one obtain an allergic patient whose symptoms are so unvarying during the twenty-four-hour period that we can tell with certainty the duration of action of a single dose of the drug. I have already referred to the difficulties in judging the sedative action of these drugs. Only by a sufficient number of cases, over a fairly extended period, with a methodical approach, and in the hands of a critical and unbiased observer can these factors be evaluated.

In our method of clinical assay, several features were embodied: (1) The patients were usually instructed to take the drug as needed rather than at set intervals. (2) In almost every instance, the action of the drug thus tried was compared, at different times in the same patient, with one or more other antihistaminic and antiallergic drugs. (3) In most instances, during the period of evaluation the patient reported to the office about twice weekly, during which visits his symptomatology for the preceding several days was discussed and judged in terms of other variables, such as the pollen or mold count. (4) Placebos and substitute drugs were sometimes given when the patient's report was open to doubt. (5) The drug was tried under various conditions, in varying doses, and by various schedules in the same patient. (6) Our final grading of the patient's results was based on the following considerations: if a patient was improved once or twice, and not improved the majority of times under more strenuous conditions, he was graded as unimproved; if the relief occurred most of the time, but was absent during special periods, such as the height of the pollen season or in the congestive post-season phase, this was set down as an improvement. The results were graded as "slight," "moderate," or "excellent," the latter indicating that the symptoms were completely removed. Most results fall into the "moderate" classification, and rarely does a patient have complete freedom of symptoms.

## IV. Individual Drugs; General Characteristics

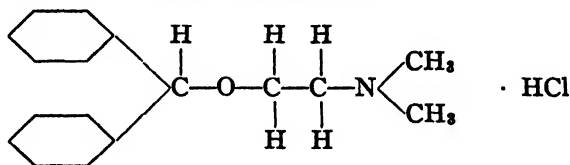
As a result of extensive experimental and clinical work with a large number of antihistaminic drugs, we have accumulated considerable data. Because of limited space and because some of the results are incomplete, I shall refrain from presenting a complete comparative report. Instead, I shall point out briefly in general terms the chief characteristics of each drug. This list of drugs, with the exception of those that were confidential at that time, consists of those with which I had experience up to 1947.

*Pyribenzamine.* (N'pyridyl-N' benzyl-N-dimethylethylenediamine HCl)  
17, 18, 22-25



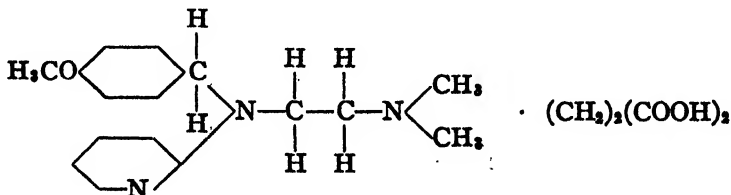
Shows potent antihistaminic action in animals, excellent action as an aerosol in guinea pigs in the prevention of histamine bronchospasm, good anti-anaphylactic activity, and excellent efficiency in inhibiting histamine whealing in man. It stands at the top of the list in clinical efficacy and reliability. Its side actions, particularly sedation, may be called average, in terms of the entire series.

*Benadryl.* (B-dimethylaminoethyl benzohydryl ether HCl)<sup>2, 3, 5, 15, 16</sup>



Antihistamine and aerosol action and antiwhealing effect good, but less striking than Pyribenzamine. Antianaphylactic action compares favorably. Clinical efficiency good, but not as effective as Pyribenzamine in nasal allergy. Sedative action is marked and frequently interferes with therapy.

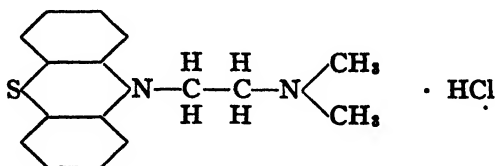
*Neoantergan.* (N-p-methoxybenzyl-N-dimethylaminoethyl  $\alpha$  aminopyridine maleate)<sup>3, 26-28</sup>



Antihistamine action is very high in terms of multiple lethal doses, but about the same as Pyribenzamine on basis of 1 MLD and aerosol method. Antiwhealing and antianaphylaxis are about the same as Pyribenzamine.

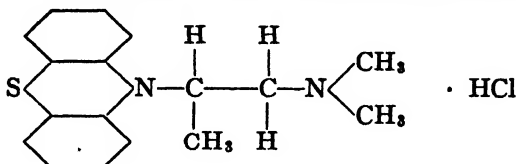
Clinical effectiveness good. Sedation moderate and gastrointestinal irritation not infrequent.

3015RP. (N-dimethylaminoethyl thiodiphenylamine HCl)<sup>29,30</sup>



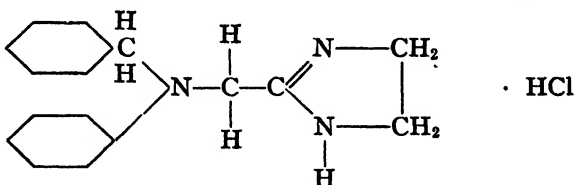
Good antihistaminic and antianaphylactic action. Poor antiwhealing effect in man. Negligible clinical response.

3277 RP. (N-dimethylaminopropyl thiodiphenylamine HCl)<sup>29,30</sup>



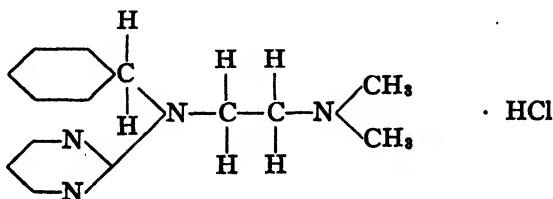
Antihistaminic effect on basis of 1 MLD<sub>100</sub> comparable to Benadryl, Pyribenzamine, and Neoantergan. Aerosol is very irritating and has poor antiwhealing effect in man. Possible clinical effect completely overshadowed by extreme sedation even in small doses.

Antistine. (2-(N-phenyl-N-benzyl-aminoethyl)-imidazoline HCl)<sup>1,31,32,33</sup>



Antihistaminic and antianaphylactic action is much less than in the other drugs mentioned above, although more complete data with larger doses of the drug are needed. Antiwhealing action is moderate. Clinical effect is moderate and requiring large doses. Toxic action in man is very slight.

NH-188. (2-(N-dimethylaminoethyl-N-p-methoxybenzyl)-aminopyrimidine HCl)

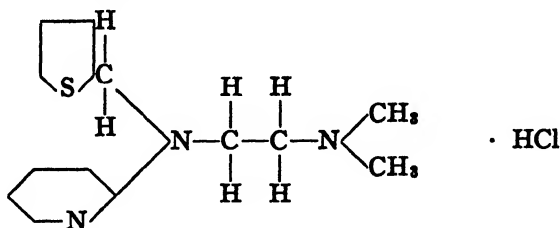


The antihistaminic, antianaphylactic, and aerosol action are very moderate. The antiwhealing property is moderate. Clinical effect is moderate, re-



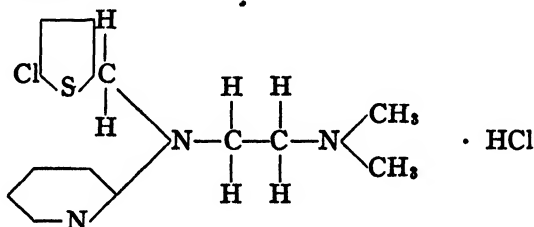
quiring larger doses than most drugs. An outstanding characteristic of this drug is its relative freedom from undesirable side actions.

*Thenylene and Histadyl.* (N-( $\alpha$ -pyridyl)-N-( $\alpha$ -thienyl)-N', N-dimethylethylenediamine HCl)<sup>1,34-36</sup>



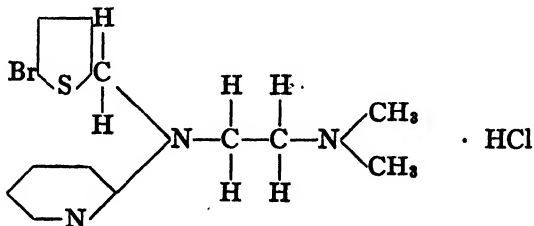
Excellent antihistaminic, aerosol, antiwhealing, and antianaphylactic effects. Good clinical effectiveness, but probably not quite so potent as Pyribenzamine. Sedative action about the same or slightly greater than Pyribenzamine.

*Chlorothen.* (N,N-dimethyl-N'-(2-pyridyl)N'-(5-chloro-2-thienyl) ethylenediamine HCl)<sup>37,38</sup>



Antihistamine action excellent. Antianaphylactic and antiwhealing effects good. Its clinical efficiency is very good, but perhaps not quite so high as Pyribenzamine. Its sedative action is about the same as or possibly slightly greater than Pyribenzamine.

*Bromothen.* (N,N-dimethyl-N'-(2-pyridyl)N'-(5-bromo-2-thienyl) ethylenediamine HCl)



The activities of this drug appear to be identical in all respects to those of Chlorothen.

### Summary

(1) The antihistaminic drugs are valuable adjuncts in the symptomatic treatment of allergic manifestations. They should not and cannot replace

more basic and more durable immunologic therapy of elimination and desensitization, nor should other antiallergic drugs be discarded.

(2) The antihistaminic drugs possess undesirable side actions, of which the most troublesome is sedation. The possible remote toxic effects of many drugs are still not ascertained.

(3) Because of differences in individual clinical and toxic response, it is of advantage to have available a choice of a reasonable number of drugs. It is obvious, however, that there is a limit to the number of such drugs needed for this purpose.

(4) Opportunity still exists for the synthesis of the ideal antihistaminic drug, which will be sufficiently potent and entirely nontoxic.

(5) The clinical evaluation of these drugs presents a complex and difficult problem and requires, at the least, the painstaking and extended effort of unbiased and astute clinical observers.

(6) Many experimental methods of assay have been tried by the author and by others. Of all the assay methods tested thus far, we believe that the antiwhealing effect on the human skin will show the greatest correlation with clinical effectiveness.

(7) The advent of the field of antihistaminic drugs has opened up new questions concerning histamine function and new approaches to experimentation in many fields, including the mechanism of the allergic reaction.

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### *Discussion of the Paper*

S. WALTER LANDAU (*The Johns Hopkins Hospital, Baltimore, Maryland*):  
Our group at The Johns Hopkins Hospital has used fifteen different anti-

histaminic drugs clinically, and has tested most of these drugs in the intestinal strips of guinea pigs and against one lethal dose of histamine injected in the bloodstream of guinea pigs. Our clinical results can be summarized as follows:

In urticaria, relief ranged from good to excellent. Results were particularly good in penicillin reactions. In many cases of atopic and contact dermatitis and in pruritus ani, the drugs were very helpful in reducing or completely controlling the itching.

In hay fever and perennial allergic rhinitis, relief was from fair to good. The mild symptoms of patients who had had beneficial hyposensitization treatment were satisfactorily controlled. Patients who had poor results with hyposensitization treatment had only slight benefit from the antihistaminic drugs.

In bronchial asthma, relief was poor. This fact should be emphasized more than has been done so far. We have seen patients with asthmatic attacks suffer unnecessarily under prolonged treatment with such drugs, when administration of adrenalin or even one of the aminophyllin compounds would have relieved them at once. We had an unusually large number of patients who had asthma late in the ragweed season in 1947. Among them were many who had previously been relieved from their hay-fever symptoms by these drugs. This time, however, they failed to derive any relief of their asthmatic symptoms.

There was no striking difference in the clinical effect of the drugs. Some patients reported more benefit from one particular preparation and some from others. Dr. Feinberg has called attention to this individual responsiveness, and this factor deserves further investigation. One might think of interference with the action of the drug in the digestive tract under certain circumstances. We have noticed, also, that some drugs act more quickly than others. Locally, we have used Antistine in the eye and have had encouraging results in a number of cases.

Our animal experiments revealed that the drugs at our disposal can be grouped into three categories of potency. However, there was no parallelism with their clinical effectiveness. This is not surprising in view of the evidence, submitted in this monograph, pointing to factors other than histamine in allergy and to effects other than antihistaminic of these compounds.

We feel that the possibility of harmful effects of these drugs has not yet been completely ruled out. When Dr. Pasteur Valery-Radot visited our clinic in September 1947, he told us that four cases of agranulocytosis due to Neoantergan have been observed in France. Such reports, the unpleasant side effects of most of the compounds, and particularly their failure to relieve serious allergic conditions such as bronchial asthma present a challenge to further thorough studies.

## GENERAL DISCUSSION\*. I

By J. A. Wells

*Northwestern University, Chicago, Illinois*

Compounds which differ widely in their stated potencies as histamine antagonists frequently have been found to be of essentially equal potency in combating anaphylaxis and certain forms of allergic disease in which the action of histamine can be presumed to be of consequence. This apparent lack of agreement in potency has been used to support the notion that actions other than histamine antagonism are responsible for the effectiveness of these compounds in allergic disease.

Basic to such argumentation is complete confidence that the original estimations of potency of these drugs as histamine antagonists are reliable and valid. There is evidence that this is not the case. Stated values for the potency of these compounds are widely different and depend upon the circumstances under which the potency was determined. Each stated value must be qualified by saying that the potency is a certain figure if one employs a particular response of a particular tissue of a particular animal to a particular concentration of histamine in the presence of a particular concentration of the antagonist.

Thus, one of the fundamental requirements in the study of antihistaminic or antiallergic drugs is a reproducible and valid estimation of potency which is independent of the circumstances under which the estimate is obtained. Such an estimation of the potency of these compounds requires some knowledge of the mechanism of their action.

The histamine antagonists fail to meet the requirements of certain of the well-known types of antagonism, such as "physiological" or "chemical" antagonism, and we have thus assumed that their mechanism of action is what has been termed by A. J. Clark as "specific" antagonism, a more descriptive name for which is "competitive inhibition." The implications of such a mechanism of antagonism are that both the active drug and its antagonist compete for the same site of attachment in a tissue, the antagonist combining with this site without eliciting a response of the tissue. In more picturesque terms, the antagonist may be considered as acting the part of the "dog in the manger."

The antihistaminics fulfill one of the requirements of competitive inhibition by their ability to antagonize the action of histamine on certain tissues from which they are incapable of eliciting a response.

The competitive aspect of this type of antagonism implies that histamine, in sufficient concentration, will overcome the effect of a given concentration of the antagonist and that the antagonist, in sufficient concentration, will overcome the effect of a given concentration of histamine. To determine this, it is necessary to investigate the quantitative response of some tissue to histamine in the presence of a histamine antagonist. For this purpose, let us consider the contraction of isolated strips of guinea-pig ileum, sub-

\* The present discussion is based upon recent work carried out in collaboration with Doctors John S. Gray and A. Zadina.

jected to the action of histamine in the presence of antagonists, such as Benadryl or Pyribenzamine.

With increasing concentrations of histamine, the responses of such strips increase greatly at first, then less and less, and finally approach a maximum. The response of this same strip to increasing concentrations of histamine can then be determined when various concentrations of the antagonist are present in the solution bathing the strip.

If one constructs free-hand curves showing the relationship between the concentration of histamine and the response at various concentrations of the antagonist, it is apparent that each curve passes through the origin and approaches the same maximum (FIGURE 1).

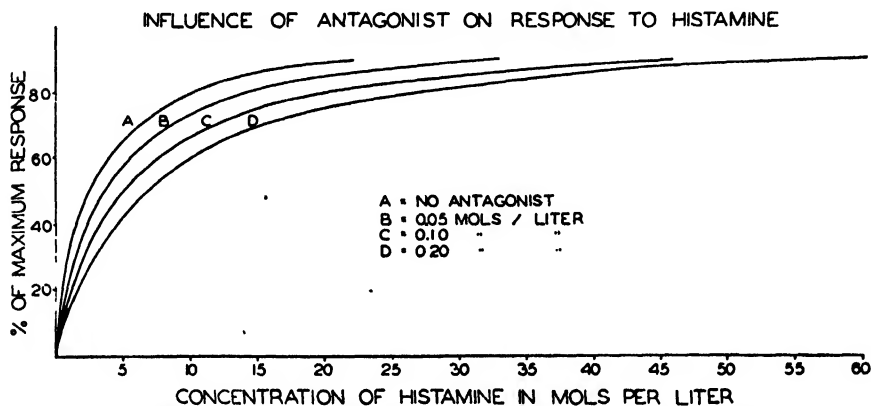


FIGURE 1. These curves are diagrammatic representations of the concentration of histamine required to cause various percentage responses of the isolated guinea-pig ileum in the presence of various concentrations of Benadryl. The various concentrations expressed as moles per liter on the graph are actually micromoles or moles  $\times 10^{-6}$  per liter.

The fact that the maximum response which can be produced by histamine is unaltered by the presence of the antagonist implies that, regardless of the concentration of the antagonist, a sufficient concentration of histamine will overcome its effects. While each curve passes through the origin and has the same maximum, they differ in curvature and tend to flatten with increasing concentration of the antagonist. This implies that, regardless of the concentration of histamine, a sufficient concentration of antagonist will decrease the response which it produces. Thus, the histamine antagonists fulfill the requirements of competitive inhibition.

Competitive inhibition implies that some sort of easily reversible union occurs between the competing drugs and a receptor in the tissue for which both have an affinity. Such a union might be of the nature of a loose chemical bond or a process of adsorption. We should, thus, look to the laws governing chemical or adsorptive equilibria for the interpretation of a system of competitive antagonists.

In line with our principal objective, it is apparent that the correct estimation of potency of the antagonist must be in terms of the affinity of the antagonist for the receptor relative to that of histamine for the receptor.

If these affinities can be quantitated, then we have a quantitative estimation of the potency. The question is: Can this be accomplished?

The first step in answering this question is to determine the type of reaction between drug and receptor. A number of possibilities exist. The antagonist, as well as histamine, may combine in mono-monomolecular fashion with the receptor, in which case only two affinities (equilibrium constants) need be determined, one for histamine and one for the antagonist. This also applies if adsorption is representative of the type of combination. On the other hand, several molecules may be involved in the reaction, in which case several equilibrium constants will need to be determined.

Solution of the problem of the type of reaction which occurs comes from the application of the law of chemical equilibria to this system of competitive antagonists. By means of such an application, it has been possible to reject the notions that competition occurs on the basis of either competitive mono-monomolecular chemical reactions or competitive adsorption. In addition, some of the more complicated types of chemical reaction may be rejected as not applying to the present system.

Let us examine the implications of the law of chemical equilibria, however, as applied to the hypothesis that histamine combines in mono-monomolecular fashion with the receptor while two molecules of the antagonist combine in stages with the receptor. Application of the law to this competitive system yields the following equation:

$$\frac{HR}{R_T} = \frac{H}{H + \alpha + \frac{\alpha}{\alpha'} B + \frac{\alpha}{\alpha' \alpha''} B^2}$$

where  $HR$  = response of the strip in mm.

$R_T$  = maximum response of strip in mm.

$H$  = concentration of histamine in mols/liter

$B$  = concentration of antagonist in mols/liter

$\alpha, \alpha', \alpha''$  = dissociation constants for the three equilibria.

This equation states that the concentration-response curves for histamine in the presence of varying concentrations of the antagonist should be a family of hyperbolas, all of which pass through the origin, have the same maximum, and differ only in their curvature, the latter being a function of the concentration of the antagonist. This relationship also states that the concentration of histamine required to produce a particular response of the strip varies in parabolic fashion with the concentration of the antagonist. Such a parabolic, constant-response isobole serves to differentiate this competitive system involving several equilibria from the simple systems involving two equilibria, in which case the isoboles are linear.

The next step is to determine quantitatively how well the experimental data conform to the equation derived from the chemical hypothesis just stated. For this purpose, correlation analysis is in order and has been performed by the statistical method of multiple correlation. The excellence of the agreement of the data with the chemical hypothesis is revealed

by the magnitude of the multiple correlation coefficients, some of which are as high as 0.99.

It is, thus, apparent that the mechanism of action of the histamine antagonists can be explained on the basis of a particular system of chemical equilibria. It is also apparent that it will be necessary to determine three affinities, one of histamine for the receptor, one of the first molecule of antagonist for the receptor, and one of the second molecule of antagonist for the receptor.

The partial regression coefficients derived from the statistical fitting provide the best estimate of the numerical values for the three affinities involved in the competitive system. Examples may be given:

$$H + R \rightleftharpoons HR \quad \frac{1}{\alpha} = 0.40 \times 10^6,$$

$$B + R \rightleftharpoons BR \quad \frac{1}{\alpha'} = 7.70 \times 10^6,$$

$$\text{and } B + BR \rightleftharpoons B_2R \quad \frac{1}{\alpha''} = 0.35 \times 10^6.$$

These values are for Benadryl, and, in a limited series, those for Pyribenzamine are of the same order of magnitude.

It may be inferred from these figures that the affinity of the first molecule of antagonist for the receptor is less than that of histamine. These affinities, while they are a function of the type of receptor, are independent of the actual responses and particular concentrations of histamine or antagonist used in their determination. Thus, their proper application should provide correct estimates of potency.

The potency of histamine, in terms of its affinity for the receptor, may be expressed as the concentration of histamine required to half-saturate the receptor in the absence of the antagonist, a value which, in the present scheme, is numerically equal to  $\alpha$ . The "potency" of the antagonist, in terms of its affinity for the receptor, may also be expressed as the concentration of antagonist necessary for half-saturation of the receptor in the absence of histamine. Since two affinities exist in the case of the antagonist, the numerical value for the half-saturation concentration in the present system

$$\text{is equal to } \frac{-\alpha'' + \sqrt{(\alpha'')^2 + 4\alpha'\alpha''}}{2}.$$

In competitive inhibition, however, the effectiveness of an antagonist is not solely a function of its affinity for the receptor, but also involves the affinity of the active drug for the receptor. Thus, the proper measure of the effectiveness of an antagonist should be based on the relative affinities of the competing compounds. The relative affinity is most logically expressed as the ratio of the half-saturation concentration of histamine to the half-saturation of the antagonist. It is proposed that this ratio, which characterizes the competitive system, rather than the antagonist alone, be designated the index of competition.



In general form this competition index is

$$\text{C. I.} = \frac{[\text{H}]_{50\%}}{[\text{B}]_{50\%}},$$

and for the particular equilibrium situation under consideration, the competition index takes the form:

$$\text{C. I.} = \frac{2\alpha}{-\alpha'' + \sqrt{(\alpha'')^2 + 4\alpha'\alpha''}}.$$

Employing the previously designated values for the various dissociation constants, the competition index for Benadryl is 20.1, which implies that, in this competitive system, the affinity of Benadryl for the receptor is 20.1 times that of histamine. It should not be forgotten that this index may be different on another tissue.

## GENERAL DISCUSSION. II

By Bret Ratner

*New York Medical College, New York, N.Y.*

I want to thank the Chairman for asking me to join in the discussion of this monograph. My first impulse would be to voice my agreement with many of the viewpoints expressed here, and to terminate my remarks at that point.

This author has, for many years, held to the view that real progress in clinical allergy would be made largely in the domain of animal experimentation. For my own part, I have probably learned more about human allergy from our studies in the guinea pig on experimental asthma, sensitization *in utero*, and passage of proteins through various living membranes than could have been arrived at from studies in the clinic.

It is well, therefore, at a conference on the subject of histamine to emphasize the importance of the immunologic basis of the hypersensitive phenomenon and bring again to our attention the three fundamental criteria of allergy: (1) the dependency upon substances known to be antigenic; (2) specificity; and (3) participation of antibodies.

Bearing these three facets in mind, namely antigenicity, specificity, and antibody participation, I might here project the problem from the lower animal to the clinic. We know that allergy is an ever present battle against the invasion into the body of foreign substances inhaled, ingested, or injected. The amelioration of allergy is based on the ability of the body to produce enough antibodies so that they may eventually reach the circulation and act as blocking or neutralizing bodies. To this end the body must continually manufacture immunizing agents. There is no single drug or substance which I can envisage as being capable of dispelling this specific antigen-antibody mechanism which is so inherent a part of the animal economy.

The hereditary atopic theory, which sought to separate human allergy from anaphylaxis in the lower animal, has been rudely brought to a halt by the observations that the atopic reagins (Prausnitz-Kustner antibodies) are found not only in the spontaneous allergies of asthma, hay fever, and urticaria occurring in man, but have also been found in the artificially induced serum sickness, *Ascaris* sensitivity, insulin sensitivity, and certain drug sensitivities, some physical allergies, as well as in hypersensitive states in the lower animal.

Animal work emphasizes anew that it is the study of the many ways and means that antigens have of invading the body and producing interactions with tissue antibodies which offer the greatest hope for good and lasting results.

Such studies entail a search for all offending substances, their elimination or reduction, and a definite program for building up tolerance. The control of environmental excesses, highly antigenic food excesses, promiscuous and thoughtless use of sera and drugs, and control of diseases which tend to

increase the permeability and dysfunction of our protecting membranes are all measures which tend to reduce the incidence of allergy.

All the papers in this monograph have emphasized the fact that the antihistaminic drugs can do no more than allay certain minor phases of allergic symptomatology. Were we to abandon the antigen-antibody hypothesis in favor of the histamine concept as the primary *modus operandi* of the allergic reaction, there would be little hope for the control and prevention of allergy.

Clinical trial of the antihistaminic drugs has now shown what they are capable of doing. They have, indeed, proved to be an enviable group of drugs that work splendidly in the alleviation of symptoms of pruritus, nasal and lachrymal secretions, and edema, but they have failed to relieve asthma or the more serious forms of dermal allergy. It is these latter manifestations, however, that comprise the largest number of allergic cases. From their mode of action, it is obvious that these antihistaminic drugs can act only as palliative agents and do not eliminate the basic mechanism responsible for allergic symptomatology.

From now on, greater emphasis should be laid on prosecuting studies in the lower animal with these antihistaminic drugs. Several groups of investigators have published some work which showed that, although antihistaminics almost uniformly prevented the intoxicating symptoms of histamine shock from occurring in the rabbit under experimental conditions, they failed to prevent specific anaphylactic shock symptoms from occurring in rabbits and guinea pigs sensitized and shocked by egg white. These authors pose the following problem: If the principal symptoms of shock and the cause of death attendant on anaphylactic shock are ascribable to the release of histamine, the amelioration of anaphylactic shock by antihistaminics should parallel their effects on histamine shock. The findings of these authors indicate that such was not the case. Whether this work will be corroborated or refuted in the future will be watched with interest.

In the light of such experiments, I should like to close my remarks by re-emphasizing the fact that the allergic manifestations of the lower animal exhibit complexities approaching, in a measure, those of the human subject, and analysis of them may uncover a wider biological meaning.

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*Editor*  
ROY WALDO MINER

*Associate Editor*  
B. J. HENEGAN

## MEDICAL MYCOLOGY\*

*Consulting Editor and Conference Chairman*  
FREDERICK REISS

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# INTRODUCTION

By Frederick Reiss

*New York University-Bellevue Medical Center, New York, N. Y.*

It is a pleasant duty and a great honor to introduce this monograph, which is the result of the first conference on medical mycology in the United States. The many authors and the diversity of their topics are indicative of the growing interest in the subject. The purpose of this monograph is not only to present recent data, but also to discuss some of the fundamental aspects of our problems which await elucidation and solution in the future.

Mycology had an earlier start than bacteriology. It continued to attract attention until the 'Seventies or 'Eighties of the last century, when the epoch-making discoveries of Pasteur and Koch in the field of infectious diseases brought bacteriology to the fore and relegated mycology to the background, where it has remained for many years. There is little doubt, on the other hand, that mycology gained an important place not only in botany and cognate technology, but also in animal and human pathology.

Though fatalities due to fungus diseases in man are not so common as those due to bacterial infections, nevertheless these mycotic diseases are important. This was recognized and emphasized by the leading medical authorities of our armed forces, in which the incidence of "athlete's foot" assumed high proportions during the last war. At Fort Benning, for instance, fungi were reported to be present in 42 to 64 per cent of the scrapings from feet. The aggravation of pre-existing fungus diseases of the skin in tropical climates was another factor which made us mindful of the importance of medical mycology. The recent epidemic of ringworm infection in scalps of school children, which had its onset during the war and is still not declining, serves as another good example of the public-health implications of mycoses. While the number of deep or fatal mycoses is relatively low (in 1942, of 1,385,187 deaths, 359 were caused by fungi in the U. S.), they equal more than half the deaths caused by the whole enteric group, tetanus, and infantile paralysis combined.

While great progress has been made in the simplification of mycological nomenclature, the classification of fungi, unlike the status in bacteriology, has not reached a satisfactory stage. As a result, the same species of fungi are described *de novo* under various new names, adding to the confusion. Also, while interesting and fundamental contributions have been made in bacteriological and virological immunity, we are still uncertain as to the interpretation of many mycological phenomena of immunity. The keratophilic tendency and affinity for dead tissues of the dermatophytes have been recognized for a long time, but the mode of their enzymatic-pathogenic action is not yet entirely clear. It is startling to note the difference in immunologic response between infections caused by fungi and fungus-like bacteria (e.g., *Mycobacterium tuberculosis* and *leprae*) and those caused by bacteria which elaborate exotoxins. Despite the fact that one of the first

polysaccharides responsible for immunological changes was isolated from trichophytons and blastomycetes, the mechanisms of immunity are not yet clarified to the same degree as they are in many bacterial infections such as tetanus, diphtheria, and botulism. In the latter group, the exotoxins are the agents of the disease, and recovery is due to the action of antibodies. No similar advance has yet been made in mycology. The treatment of superficial mycoses with fatty acids signifies a considerable step forward, but, since a large number of patients resist this therapy, a search should be made for such metabolic and immunological changes in the affected structures as might explain the therapeutic failures of many cases. New antibiotics, but mainly better penetrating vehicles, may contribute to the solution of this problem.

Neither the mode of transmission of the superficial mycoses nor their prevention is fully understood, whereas the prevention of plant mycoses has been suitably accomplished. Prophylactic vaccination, if perfected, would be just as desirable against fungus diseases as it is against diphtheria, tetanus, and smallpox.

While little difficulty is encountered in the diagnosis of deep mycoses, we are still lagging behind in the cure of most of these diseases. Penicillin appears to be a valuable drug in the treatment of actinomycosis, just as the partial desensitization in blastomycoses and subsequent treatment with iodides are helpful. None of these, however, has eliminated the seriousness of the prognosis of both diseases. Lack of familiarity of the profession with mycoses was the source of diagnostic and survey errors in relation to tuberculosis. Only in recent years has it been recognized that pulmonary calcification may be the result of infections other than tuberculosis, namely coccidioidomycosis and histoplasmosis. The treatment of coccidioidomycosis and torulosis is still most unsatisfactory, and both are generally fatal. While, in coccidioidomycosis, the epidemiological importance of wild rodents has been accepted as a contributing factor, nothing is known of the source of the transmission of histoplasmosis, nor has an acceptable explanation been offered as to why certain apparently healthy individuals react strongly to histoplasmin. The cross-reaction to blastomycin also awaits further clarification.

These are only a few of the many biological and therapeutic complexities with which we are confronted and which provide an impetus and challenge for further investigations. I am fully convinced that we are entering a fertile field which will bear a rich harvest. All those concerned must recognize the progress which has been made in this country in mycological research, and it is hoped that this may lead to even greater advances. This monograph will have served a useful purpose if it provides a stimulus to new ideas in the solution of many problems.

I wish to express my gratitude to The New York Academy of Sciences, and particularly to its able Executive Director, Mrs. Eunice Thomas Miner, for having organized this program so efficiently and for making publication of this monograph possible. I wish also to thank all the participants for their kind support, which is the augury of success.

# MYCOLOGICAL RESEARCH AND THE PROGRESS OF MEDICINE

By Carroll W. Dodge

*Henry Shaw School of Botany, Washington University, and Missouri Botanical Garden, St. Louis, Mo.*

The relation of certain fungi to human disease was early recognized, especially in the period from 1839 to 1853 in the work of Schoenlein and of Gruby, culminating in Robin's (1853) survey of fungi then associated with human disease. This early period was followed by a period of little activity, while the medical profession turned their attention to bacterial diseases. Activity was renewed about 1890, when Rénon (1897) thoroughly investigated aspergillosis. About the same time, Sabouraud began his work on the dermatophytes, which culminated in his exhaustive treatise in 1919. American work began a few years later on coccidioidal granuloma and blastomycosis (1892-1910), with renewed activity after 1930. The foundation of our knowledge of sporotrichosis was laid by de Beurman and Gougerot (1912). Actinomycosis, maduromycosis, and chromomycosis were outlined between 1890 and 1920, although much important work has been done since. Castellani began to study the imperfect yeasts about 1905, developing his taxonomy on biochemical activity. Langeron and his students placed their classification upon a much sounder basis of morphology in the 1920's, culminating in the taxonomic works of Stelling-Dekker (1931), Lodder (1934), Diddens and Lodder (1942), and Dodge (1935). Grigorakis and Moore had carried out extensive cytological investigations in the decade 1925-1935.

By 1935, the principal fungi associated with human disease had been described. Since that time, we have largely been filling in details, rather neglecting the morphology and cytology of the organisms and turning our attention to pathology and therapeutics and to the nutrition of the fungi. The importance of other groups of fungi as allergens has been studied increasingly in the last quarter century.

Research in other fields of mycology so far has had little influence on the medical field, but perhaps, in passing, I may mention lines of investigation, still in their early stages, which later may influence our thought. Baker (1944) has provided cytologic evidence for heterocaryosis in the imperfect fungi and a cytologic basis for inheritance in the absence of the usual type of the sexual act. The current studies of Lindegren and co-workers on the cytology and genetics of yeasts may give us a better idea of stable and variable characters for a better system of classification, while his work on adaptive enzymes may give a better appreciation of enzyme activity and help explain the anomalies which every worker encounters in the study of fermentation. Such studies may eventually help us to clear up the confusion created by Castellani in describing only the biochemical characters of so many imperfect yeasts.

In looking forward to fields for further research, in many cases we find



two or more organisms isolated from different cases of a clinically similar disease. We need better clinical description and differentiation, correlated with a careful study of the organism isolated in each case, before we can assume that the same clinical entity is produced by several different fungi. A notable example of the work already done in this field is that of the clinical forms of South American blastomycosis, where, in the early days, two distinct clinical forms were treated as a single entity without a careful study of the organisms. When a careful correlation of clinical types and fungi isolated was made, each organism was found to be associated with a distinct clinical type. The field of therapeutics and possible development of antibiotics will only be mentioned, as it will be treated much more fully in later papers.

We need more appreciation of the relations of vitamin nutrition and the activity of the endocrine systems of the host, especially in the field of dermatology. Although it has been long recognized that changes in the endocrine system at puberty produce corresponding changes in the skin (Greenwood and Swartz outlined such a study to me in private conversations a quarter of a century ago), it was fully twenty years before research along these lines, entirely independently, showed changes in the oil secretion and developed promising new treatments with fatty acids. Such research is still in an early stage and we can expect important developments along these lines.

While in Guatemala in 1941-1942, I had an opportunity to observe many cases of a partial achromia, mostly on the exposed portions (face, neck, and forearms, and sometimes also on the legs) of prepubertal males. Quite regularly, from the very slight scaling over the partially depigmented areas, I isolated *Hormodendrum fontoynti* Langeron or a very closely related species, reported by Fontoynt and Carougeau (1922) from a similar disease, hodi potsy, in Madagascar. Occasionally, I also isolated the same organism from scales not accompanied by the partial achromia, and I was inclined to consider the *Hormodendrum* a skin saprophyte. Shortly before I left Guatemala, a medical friend treated his son for an undescended testis. For some months, I had observed in the son a very characteristic case of the partial achromia. Within two weeks after the treatment, scaling disappeared and the pigmentation became wholly normal. After this observation, the father and I planned to study and treat selected cases in the school clinics to see whether there was a definite relation between the partial achromia and the gonadal hormones. Unfortunately, it was at the height of the submarine activity in the Caribbean, which had completely interrupted commerce, and the War Department was utilizing the total freight capacity of the airlines, so there was practically no supply of the hormones in the country. I had to return shortly afterward and the matter was dropped without our arriving at any satisfactory conclusion.

While in Guatemala, I also had the good fortune to study several cases of a disease closely resembling, if not identical with, tokelau, in a small area along the road between Lake Atitlan and Patulul. This disease had already been studied by Herrera Solis (1932), and a recent single case was reported by Figueroa and Conant (1940). Frequently, *Endodermophyton*

has been isolated from the characteristic scales of these patients. The scales from the patients were carefully collected in sterile petri dishes and blood samples were taken for Wassermann reactions, as some of Herrera Solis's patients had been Wassermann positive. All samples were Wassermann negative, ruling out any possible syphilides. Microscopic examination of the scales showed fungus hyphae of the ringworm type.

From twelve cases, I isolated *Endodermophyton* in two cases, *Epidermophyton* in two cases, *Ectotrichophyton* in only four cases. In two prepubertal boys, I isolated only imperfect yeasts. Two other cases, not so clinically typical, yielded *Ectotrichophyton*. One of the *Endodermophyton* cases also yielded a colony of an *Ectotrichophyton*.

On reviewing my clinical notes, I found that all the patients, except one prepubertal boy, were conspicuously undernourished. Herrera Solis had noted the same condition in his eight cases. Normals examined in the same small community were well nourished, as is general in that part of Guatemala. Since vitamin A seems to affect the condition of the skin more than the other vitamins, it seems likely that the lack of this vitamin had so altered the outer horny layer of the skin that the *Endodermophyton* and other skin saprophytes had been able to grow more luxuriantly than on normal skin and had contributed to the characteristic scaling. While I observed only one case of obvious endocrine dysfunction (an elderly woman with a large goiter) in these patients, it may have been a complicating factor, as varying degrees of thyroid dysfunction are said to be common in this area.

We should also recognize the possibility in fungus infections of a symbiosis of two or more organisms, perhaps complicated by endocrine dysfunction, especially at the thyroid, as seems to be the case in seborrhea and acne.

Another question has intrigued me for some time. Why are some organisms more invasive in some individuals than in others? As an example, I may cite two successive cases of *tinea cruris* observed in Guatemala. The first was a sailor, nervous from the submarine sinkings he had witnessed in the Caribbean during the previous few weeks. The lesions, which had developed in about a week, occupied the gluteal fold, nearly covered the buttocks, the perineum, the inner aspects of the thighs half way to the knees, and followed the pubic hair tract nearly to the umbilicus. The pruritus was intense and he was continually walking the floor of his room. He showed considerable self-control, however, as there was little excoriation and no secondary infection. He was a blond with very fair skin and little body hair. The next case was a placid Guatemalan business man. The lesion, which had been developing for about a month, was a typical round plaque, about 6 cm. in diameter, on the inner aspect of the thigh opposite the scrotum. The patient did not complain of pruritus, although there was some excoriation. He was a brunet with a darker skin than the first patient, but much lighter than the average Guatemalan. The body hair was abundant over the trunk and thighs. Both seemed equally well nourished without endocrine dysfunction. Physical factors of the environment were essentially similar, as the Guatemalan's business kept him in the tropical lowlands

most of the time. Had the nervous excitement of the sailor so altered the outer layer of the skin that the fungus had found conditions for invasion and growth more favorable?

We need more study of the saprophytic phase in the life cycles of pathogenic fungi. Where does the fungus vegetate between human cases? Are there subclinical cases which keep the organism going between the severer, recognized cases? How are the organisms transmitted? These are some of the questions which need prompt solution. Yet, except in the case of the dermatophytes, we have very little information, and even with the dermatophytes there is much more to be learned.

Finally, we need more monographic studies, including the saprophytic as well as the parasitic species in the groups concerned, embracing the morphology, cytology, and physiology, especially in the imperfect yeasts and the groups with dark-colored mycelium, such as the *Dematium-Hormodendrum* series.

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## PUBLIC HEALTH ASPECTS OF FUNGUS INFECTIONS

By S. B. Salvin

*Division of Infectious Diseases, National Institutes of Health, Bethesda, Maryland*

Fungus infections in man, although less frequent than bacterial, are still numerically important. For example, of the 92,933 deaths due to infections and parasitic diseases in the United States in 1945, 284, or 0.3 per cent, were due to mycoses.<sup>1</sup> This was approximately equal to the number of deaths reported as caused by scarlet fever, measles, or the typhuslike diseases (due to rickettsia), and was more than the total of all deaths recorded from rabies, smallpox, relapsing fever, leprosy, brucellosis, paratyphoid fever, plague, cholera, and anthrax. It should be realized, of course, that effective control measures are employed against some of the aforementioned diseases, whereas control methods against the mycoses not only are not practiced, but, generally, are not even known. It should also be borne in mind that the dermatophytoses, although characteristically nonfatal, are extremely common, probably equaling the most widespread of the bacterial or virus diseases in prevalence.

Many species of fungi have been characterized as having produced disease in man and the higher animals. Some, such as *Blastomyces brasiliensis* and *Blastomyces dermatitidis*, have been isolated only from the parasitic state. Others, such as *Aspergillus fumigatus* and *Sporotrichum schencki*, have been demonstrated as existing in nature both as pathogens and as saprophytes. Indeed, it is often difficult in some instances to determine whether the role the fungus plays within a given host involves a parasitic or saprophytic existence.

The association of a potentially pathogenic fungus with man does not necessarily imply that parasitism exists. The frequent isolation of *Candida albicans* from the intestinal tract of cases of sprue precipitated the erroneous belief that that fungus was the etiologic agent for the disease. Similarly, the frequent association of various species of *Aspergillus*, *Penicillium*, etc., with pinta pointed toward these fungi as the causative organisms. Repeated demonstration of the fungus in the host and its isolation therefrom, to be followed by the development of disease in experimentally infected animals and by the subsequent recovery of the causative fungus, are often necessary to prove the pathogenic role of an organism.

The presence of these sometime pathogens in pus, skin lesions, or sputum should be weighed very carefully before they are assigned etiological significance. For example, *Candida albicans* is often isolated from sputum, especially if the sputum is permitted to remain at room temperature for several hours, under which circumstance rapid multiplication of the fungus occurs. Thus, examination of old sputum may produce an erroneous picture of the true mycologic flora. Even the isolation of this species from fresh sputum does not warrant a diagnosis of moniliasis, for Benham and Hopkins<sup>2</sup> found the fungus on 6 per cent of normal tongues, Todd<sup>3</sup> in the throat and mouth of 14 per cent of 1000 normal persons, and Knighton<sup>4</sup> in 24 per cent of 146

normal mouths. No correlation was found between the presence of *C. albicans* and dental caries or diseases of the gums. Up to date no practical method has been presented for evaluating properly this occasionally pathogenic, but often apparently noninjurious, fungus in sputum from unidentified pulmonary disease.

There are four deep mycoses of public health interest which will be discussed here in some detail, with emphasis on their geographic distribution, their natural habitat, their mode of transmission, and their mode of infection. The first is actinomycosis, the common type of which, caused by the anaerobic *Actinomyces bovis*, is world-wide in distribution and doubtless is the most common of the deep mycoses.

Actinomycosis was first described in cattle by Bolinger in 1876,<sup>5</sup> although the organism, named by Harz<sup>6</sup> in 1877, was not isolated in culture, studied, and described until 1891, by Wolff and Israel.<sup>7</sup> At the same time, Bostroem,<sup>8</sup> using aerobic methods for cultivation, isolated an aerobic species, probably a *Streptomyces*, which he erroneously described as *A. bovis*. This isolate is now generally considered a chance contaminant, but its being a type common to soil gave rise to the popular misconception that *A. bovis* occurs in nature on grasses and that it is transmitted from such vegetation to man and animals. Actually, organisms of the Wolff-Israel type have not been isolated from outside the human or animal body, and, probably, do not thrive ordinarily in the outside world because of their inability to grow at low temperatures, their need for reduced oxygen tension, and their lack of spores.

The claim that Bostroem's organism caused actinomycosis was partly responsible for the belief that actinomycosis is primarily a disease of rural groups, transmitted either by contact with infected animals or by traumatization with infected grasses, straws, or grains. Davis<sup>9</sup> examined 46 new cases with regard to this belief, and found that, in general, rural workers were no more susceptible to infection than urban individuals. Of the 46 cases, only 15 were farmers, with but 3 having a history of chewing grasses. In addition, no definite instance has been recorded where the disease has been passed from animal to man, although suggestions of such transmission may be found. Griffith<sup>10</sup> found club-bearing granules in beef tongues and other tissues. Although actinobacillosis was primarily responsible for this condition, true actinomycetes were also present. Nevertheless, no case of actinomycosis in man has been demonstrated as resulting from the ingestion of such infected food. Baracz<sup>11</sup> and McKenty<sup>12</sup> reported cases of possible transmission of the disease from man to man, but the evidence is not very convincing.

The early studies of Wright<sup>13</sup> and Lord<sup>14, 15</sup> demonstrated that *A. bovis* may be found in carious teeth. Lord also isolated the organism from tonsillar crypts of patients without actinomycosis, work later confirmed by Emmons<sup>16</sup> and by Slack.<sup>17</sup> The potentially pathogenic *A. bovis* may thus occur in small numbers as part of the flora of the normal mouth, and, together with the lactobacilli and the oral spirochetes, make the oral and pharyngeal mucous membranes its natural habitat.

The method whereby this fungus becomes transformed into a destructive parasite in adjacent or remote tissues is still a matter of conjecture. Local trauma, although possibly contributory, is not the main causative factor, as indicated by the relative infrequency of the infection in man. It is interesting to note, however, that a history of tooth extraction or an injury to the mouth has been recorded in cervico-facial actinomycosis,<sup>18</sup> and that instances of actinomycosis in the hand have followed punctures of the skin by human teeth.<sup>19-21</sup> According to Lord, imperfect drainage of a carious tooth, whether arising spontaneously or as the result of filling an incompletely sterilized tooth, may be regarded as favorable to the production of disease about the jaws and neck, the root canal of the tooth being the channel of infection.<sup>22</sup>

Finally, the probable role of *A. bovis* in tartar formation suggests that such calculi may constitute a major part in the pathogenesis of actinomycosis. Naeslund<sup>23,24</sup> reported that the three clinical types of tartar—supragingival and subgingival calculus and salivary duct stones—always contain a stroma of *Actinomyces* and *Leptotrichia* vegetations, with typical actinomycotic clubs occasionally demonstrable. He isolated anaerobic strains repeatedly from the calculi, and succeeded in producing concretions *in vitro* with pure cultures. The theory has therefore been proposed that calculus is produced by the activity of *Actinomyces* and to some extent by *Leptotrichia*, both of which induce the precipitation of salts from the saliva and also serve as a matrix to attach the tartar to the tooth surface. Since the microorganisms stay alive at the surface of the calcification, the process is progressive rather than self-limited.

This occurrence of viable *Actinomyces* in tartar led Colebrook<sup>25</sup> to suggest that fragments of such calculus may become detached from the teeth, and, on becoming impacted in tissue, initiate progressive actinomycosis. Although there is no direct evidence for this view, it is logical, especially since Slack<sup>17</sup> and Rosebury *et al.*<sup>26</sup> succeeded in producing progressive actinomycosis experimentally in animals with strains of *A. bovis* isolated from pyorrhea pus and gingival scrapings, respectively.

Another one of the more frequent mycoses, and one especially interesting because of its occupational relationship and the peculiar diagnostic problems involved therein, is sporotrichosis, first recognized by Schenck<sup>27</sup> in this country in 1898, and shortly afterwards by De Beurmann in France.<sup>28</sup> It has since been reported from all the continents, although a preponderance of the reported cases stem from France, the United States, and South America. There is some indication of an endemic tendency, since five-sixths of the 57 cases cited by Ruediger<sup>29</sup> were from the Missouri Valley, and 130 out of the 148 cited by Foerster<sup>30</sup> were from the Mississippi River basin, primarily, certain localities in the Dakotas, Nebraska, Wisconsin, Kansas, and Missouri. This distribution may suggest a preference of the fungus for certain types of vegetation and for particular climatic conditions.

Of 18 cases described by Foerster,<sup>30</sup> 14 were employees of a tree nursery, and at least 10 undoubtedly acquired the infection by traumatization with the thorns of the barberry shrub, primarily the Japanese barberry, *Berberis*

*thunbergii*. In two of these cases, barberry thorns were removed from the primary lesions after suppuration had started; in 6, deeply embedded barberry thorns had been removed from a few days to two weeks prior to the development of sporotrichotic lesions at those sites; in 2 others, the patients had repeatedly pricked themselves with the thorns preceding the appearance of the disease. St. Girons<sup>30</sup> in France also reported several cases in which the barberry seemed to be the source of infection. Wakefield observed a series of cases in gardeners in the vicinity of Chicago, while numbers of florists have been reported victims of the disease. Emmons<sup>31</sup> isolated the fungus from sphagnum moss, which was responsible for several cases in florists. The fungus was also obtained from timbers of a mine in South Africa, and from the miners exposed to this material.<sup>32</sup> These and the preponderance of cases among rural inhabitants and others close to the soil suggest that the favorite habitats of *Sporotrichum schencki* are probably shrubs, grasses, and the like, although bushes with thorns are more apt to convey the disease to humans.

Benham and Kesten<sup>33</sup> described the saprophytic growth of the fungus on experimentally inoculated barberry thorns and in carnation buds, and showed that the fungus retained its virulence for animals after a brief saprophytic life on plants.

Sporotrichosis has also been reported in lower animals,<sup>34</sup> appearing spontaneously in horses and livestock in certain parts of the United States. However, the relative absence of the disease among the veterinarians and workers in the areas where equine sporotrichosis is common indicates the unimportance of this channel of contact. Ruediger<sup>29</sup> reported one case in which the initial lesion of the disease appeared on the site of a recent hen bite, and another in which the first nodule developed on the site of a wound from a wire in a barn containing a sporotrichotic horse. The disease has been described as occurring spontaneously in dogs, rats, rabbits, cats, and other small mammals.<sup>35-40</sup>

The third mycosis of interest here is coccidioidomycosis. Originally, this disease was believed to be limited to the Chaco region of Argentina<sup>41, 42</sup> and to the San Joaquin Valley in California,<sup>43</sup> but recent work by Phillips,<sup>44</sup> Smith,<sup>45</sup> and others<sup>46-50</sup> has shown the fungus to be present throughout the arid Southwest, in southern California, southern Utah, Arizona, New Mexico, and west Texas. This mycosis has recently assumed added significance because of the large number of soldiers who were stationed in the endemic area and the many tourists who pass through. Each individual in these groups, on developing an acute respiratory infection, becomes another possible case of coccidioidomycosis with its added diagnostic and therapeutic problems.

The infection by *Coccidioides immitis* is evidently not transmitted directly from one individual to the next; thus, there is no reason for isolation of the patient. The parasitic or spherule phase of the fungus which appears in human sputum or pus is infectious when introduced experimentally into laboratory animals,<sup>51</sup> but seemingly is of no importance in the natural direct transmission of the ailment. The chlamydospores from the saprophytic

phase of the fungus are the main infectious agents. Epidemiological studies, the clinical picture, the pathology of disseminated infections, accidental laboratory infections, and experimental production of the disease in guinea pigs by inhalation point toward the probability of infection by inhalation of air-borne spores. There is a good correlation of exposures to dust storms and onset of symptoms, with the result that the spores were assumed to be present in the dust and the fungus to be thriving in the soil from which the dust arose.

Little success has been evident, however, in attempts to isolate *Coccidioides* from the soil. The fungus was isolated first from the soil near a Delano, California, ranch house where there were four cases of coccidioidal granuloma;<sup>52</sup> then in Panoche Valley, California, near a burrow from which a group had dug a rattlesnake;<sup>53</sup> and, thirdly, from five (out of 150) soil samples collected on the desert near the village of San Carlos, Arizona.<sup>54</sup> Hundreds of other attempts to isolate *Coccidioides* from the soil of endemic areas have failed.

Recent studies, however, have presented the possibility of a rodent reservoir of *Coccidioides*. Of 303 animals from an endemic area, trapped and examined by Emmons<sup>55</sup> for possible pathogenic fungi, 128 were infected: 16 with *Coccidioides immitis* alone; 9 with both *C. immitis* and *Haplosporangium parvum*; and 92 with only *H. parvum*. The types of animals found infected were the pocket mouse, *Perognathus*; the grasshopper mouse, *Onychomys*; the kangaroo rat, *Dipodomys*; and a ground squirrel, *Citellus*; with the pocket mouse (*Perognathus*) and the kangaroo rat (*Dipodomys*) showing the highest percentages of natural infection, namely, 15 per cent and 17 per cent, respectively. This second fungus, *Haplosporangium parvum*, which, in some respects, resembles *Coccidioides* in the parasitic phase, causes a somewhat similar pulmonary disease in rodents, but has not yet been recognized as an agent in human disease.

Coccidioidomycosis in the rodents apparently was a chronic disease which did not seem to interfere with the normal reproduction and development of the animals. It was suggested that the fungus may be primarily a pathogen of rodents and present in soil that has been contaminated by infected rodents.

The diagnosis of coccidioidomycosis in humans depends upon the demonstration of the fungus in pus, sputum, or tissues. The coccidioidin skin tests and serological tests have assisted greatly, however, in adding to the knowledge of the disease. Those who have had primary coccidioidomycosis, even in clinically inapparent form, retain a skin sensitivity for many years.

Aronson, Saylor, and Parr,<sup>56</sup> in a study of calcified pulmonary nodules in tuberculin negative individuals, found a very high percentage of positive reactions to coccidioidin in people living within the endemic areas of coccidioidomycosis, and virtually no reactions in other nonresident groups. They concluded that some of these calcifications may be due to *Coccidioides*.

C. E. Smith<sup>56</sup> reported that the first symptoms appeared 7 to 21 days after inhalation of the chlamydo spores, and sensitivity to coccidioidin in 10 to 45 days. His survey of the San Joaquin Valley cases over a period



of 17 months illustrated the association between the onset of disease, field work by the inhabitants, and the presence of dust.

The seasonal variation of the disease and its association with dust were again emphasized in studies by C. E. Smith *et al.*<sup>57</sup> at four Army airfields in the San Joaquin Valley during the last war. In the fall of 1941, when Minter and Gardner Fields were being built, there were extensive scarred surfaces and much dust. That season of maximal dust provided the highest coccidioidal rates of the study. The following year, lawns were planted, roads were paved, airstrips hard-surfaced, and the incidence of coccidioidal infections was cut in half. When the fields were oiled in June, 1944, the incidence was lowest in their history.

Finally, among the deep mycoses, we come to histoplasmosis, a disease originally described in Panama in 1906,<sup>58, 59</sup> but since then reported from the United States,<sup>60</sup> South America,<sup>61</sup> Java,<sup>62</sup> England,<sup>63</sup> Philippine Islands,<sup>64</sup> Germany,<sup>65</sup> Turkey,<sup>66</sup> and several other areas.<sup>67</sup> The disease is sporadic in its occurrence, with no distinct indication of its having been transmitted from person to person, although McLeod *et al.*<sup>68</sup> did report two cases that had had contact with one another. The fungus has been isolated in culture from spontaneously infected dogs,<sup>69-74</sup> mice, and rats,<sup>75</sup> which animals therefore may be possible reservoirs of the disease.

Although only slightly more than one hundred cases have been reported in which the organism, *Histoplasma capsulatum*, has been found, the works of Christie and Peterson,<sup>76-78</sup> Palmer,<sup>79-80</sup> and others<sup>81-83</sup> have brought forth the suggestion that there is a mild, quite common form of histoplasmosis. Palmer,<sup>79</sup> for example, after a study of 3000 student nurses, concluded "(a) that mild, probably subclinical infection with *H. capsulatum* (or an immunologically related organism) is widely prevalent in certain states and relatively infrequent in others; (b) that, in general, those states in which the frequency of reactions to histoplasmin is high are those in which pulmonary calcifications observed is also high; (c) that a high proportion of the pulmonary calcifications observed in roentgenograms of tuberculin negative persons is due, not to tuberculosis, but probably to histoplasmosis."

This conclusion is drawn from the results of skin tests with histoplasmin, a cell-free filtrate of a broth in which *H. capsulatum* has grown, and from the reported correlation between sensitivity to histoplasmin and the occurrence of pulmonary calcification. Emmons *et al.*,<sup>84</sup> however, showed that histoplasmin elicited positive reactions in guinea pigs with experimental histoplasmosis, blastomycosis, coccidioidomycosis, haplomyces, and moniliasis. The rate of pulmonary calcification is high in east-central United States and seems to be caused by some disease other than tuberculosis, although the exact causes of nontuberculous pulmonary calcification still are in doubt. Proof that histoplasmosis is one of the etiologic agents of this condition awaits the use of a specific diagnostic procedure and the isolation of *Histoplasma* from the hypothesized frequent mild cases of the disease.

Probably one of the most troublesome public health problems of the past few years was ringworm of the scalp, caused by *Microsporum audouinii*. *M.*

*canis*, and several species of *Trichophyton*. Several epidemic outbreaks of tinea capitis in large cities, chiefly in the eastern parts of this country, have been recorded.

Montgomery, in a discussion after the paper of Lewis *et al.*,<sup>85</sup> reported an average of 77 cases of tinea capitis per year from 1935 to 1942 at a New York City clinic, with 47.4 per cent caused by *M. audouini*. In 1943, at the same clinic, the total number of cases increased greatly to 572, with 86.7 per cent caused by *M. audouini*. This increase in the percentage of cases caused by *M. audouini* and its appearance in epidemic proportions probably were partly the cause of its being more contagious, more resistant to treatment, and better adapted for human infection than *M. canis*. Benedek and Felscher<sup>86</sup> reported 140 cases in Chicago, from 1940 to 1942, of which 81.5 per cent were caused by *M. audouini* and 12.2 per cent by *M. canis*. Livingood and Pillsbury<sup>87</sup> reported 130 cases in Philadelphia in 1941, of which 96.2 per cent were caused by *M. audouini*. Miller, Lowenfish, and Beattie<sup>88</sup> reported 928 new cases from 1943 to 1945, of which 96.9 per cent were due to *M. audouini*. Schwartz *et al.*<sup>89</sup> reported 565 out of 8,657 children infected in their survey at Hagerstown, Maryland, with all but 8 caused by *M. audouini*. Carrick,<sup>90</sup> in a survey of 3565 Detroit elementary-school children chosen at random, found 2.7 per cent with signs of infection as indicated by Wood's light. On the basis of the total number of children subject to ringworm of the scalp, he estimated that there were about 6000 cases of tinea capitis in the Detroit public schools. Lewis *et al.*<sup>85</sup> believed that these recent outbreaks resulted primarily from the decreased maternal care and supervision during the war, the movement of infected children from place to place because of reasons connected with the war effort, the crowding of institutions for children, and the concomitant inefficient supervision.

Among the more important steps for prevention and control are the early recognition and reporting of the disease. Isolation and early adequate treatment are necessary to prevent spread to other children and to other areas of the body of the same individual. Most dermatologists believe that infected children should be excluded from school until recovery, and, in institutions, the infected should be separated from the healthy. Each infected child should be provided with a stocking cap or some similar head covering that can be burned after use. All contacts with infected children should be examined with Wood's light, a piece of apparatus that should be available at the local school or health department. Educational programs should be pursued for parents in epidemic areas.

Schwartz *et al.*,<sup>89</sup> in their studies at Hagerstown, found 12.1 per cent of the boys infected and only 2.1 per cent of the girls. Apparently, the boys and girls had equal exposures in schools, playgrounds, homes, and movies, but not in the barber shops, which were attended by nearly all the boys but by only a few girls. Examination of the barber shops revealed generally poor sanitary conditions, with infected hairs found frequently in the electric clippers. Of the infected boys, 65 per cent had the fungus present in the clipper area only, 31 per cent over the head, and 4 per cent on the crown

of the head only. In the girls, the fungus was present always in the area of the hair-part.

According to Lee,<sup>91</sup> the results of a questionnaire to state health departments in August, 1946, showed that ringworm of the scalp was a reportable disease in three states only, Illinois, Ohio, and Pennsylvania. The city health departments of St. Louis, Cleveland, and Philadelphia, however, required the reporting of this infection. In Jersey City and Newark, tinea capitis is categorized as a public health problem, but reporting the disease is not required by law.

The most common of all the fungus diseases are the dermatophytoses, caused by *Epidermophyton floccosum* and by various species of *Trichophyton*. Among these, the incidence of dermatophytosis of the foot is probably the highest. For example, Legge, Bonar, and Templeton<sup>92</sup> found that 51.5 per cent of the men admitted to the University of California during a single year had "athlete's foot," and that the percentage increased to 78.6 by the end of the year. Alderson and Reich<sup>93</sup> reported that dermatophytosis of the foot represented 24.7 per cent of the skin diseases in a student health service. Lomholt<sup>94</sup> reported that the incidence by clinical diagnosis in school children in Copenhagen was almost 50 per cent. Of 354 men surveyed in an industrial plant,<sup>95</sup> 36.4 per cent were found by cultural examination to be infected.

Many compounds and methods have been used in the attempt to control these skin pathogens. One of the more common ones involved the use of 1 per cent sodium hypochlorite in foot baths, in spite of the fact that Bonar and Dreyer<sup>96</sup> found the fungi in skin scales still alive after one hour's exposure to the hypochlorite. Dermatophytes were found to flourish on old floors, but not on clean sound wood. The fungi were found to remain alive on shoe leather for months and to grow on stockings,<sup>97</sup> but were killed on fumigation with formaldehyde.<sup>98, 99</sup> Bonar and Dreyer<sup>96</sup> demonstrated that standard power laundry techniques kill the dermatophytes, primarily because of the high temperatures used, although standard dry-cleaning solvents and ordinary home laundering are not fungicidal.

Finally, mention should be made of a possible role of dermatophytes, one not yet proven but, if true, one of great importance and interest, namely, the part of dermatophytes in eliciting peripheral vascular diseases. Thompson<sup>100, 101</sup> stated that one-third of the admissions to the surgical dispensary were inflammatory disorders of the peripheral vascular system, the lesions of which are characterized by endothelial necrosis and inflammation, thrombosis, and by painful ulcerations or gangrene of extremities. These lesions he considered as possibly being aroused by the inflammatory action of the products of some of the dermatophytes. If true, this role should increase still further the importance of the dermatophytes as a public health problem.

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# EVALUATION OF CLASSIFICATION OF PATHOGENIC FUNGI

By Morris Moore

*The Barnard Free Skin and Cancer Hospital; the Barnes Hospital; and the Department of Dermatology, Washington University School of Medicine, St. Louis, Missouri*

The necessity for classifying pathogenic fungi is no less urgent or important than in the cases of plant life in general and animal life. The confusion existing in man-made attempts at the orderly arrangement in a phyletic scheme of pathogenic fungi, however, has made it essential, more than ever, that such a classification be clearly defined and established.

To evaluate properly the classification of pathogenic fungi, it would be in order to set forth the criteria by which the taxonomist is necessarily governed in arriving at a justifiable classification. Of prime importance is morphology. The recognition of the various forms and cell structures which serve to make up the organism and consequently relegate it to an established genus and species is a chief factor. Much depends on the interpretation of cell forms, especially in the absence of the perfect structure, the result of the sexual act. The size and shape of a specific cell structure, such as the macroconidium or fuseau, is used by some as a means of differentiating the genera *Microsporum*, *Trichophyton*, and *Epidermophyton* (FIGURE 1).<sup>1</sup> Where the perfect form is present, and this is a rarity with pathogenic fungi, the classification of the fungus is relatively simple. Since most of the pathogenic fungi are members of the Fungi Imperfecti, classification depends on morphologic characteristics, including methods of spore formation, types of cell structures, location of spores, and special organs. As important as morphology is, however, variations may result from alteration of environmental or physical phenomena and consequently must be viewed and interpreted with caution.

Gross cultural characteristics, like microscopic morphology, play an important role in diagnostic procedure. Size, shape, color, texture, and surface markings are of value in the macroscopic determination of fungi. As in the case of microscopic morphology, gross cultural characteristics may also be altered perceptibly by external factors, which should be considered by the investigator.

In a study of pathogenic fungi, with classification in view, efforts are directed towards a determination, if possible, of the perfect stage, the sexual act, and the resulting structure. To this end, the fungus is grown on a medium which might prove to be suitable for such a determination. Altered physiologic response of the fungi due to environmental factors plays important roles in such a procedure.

What are some of these factors? To name a few, we have various nutritional substances, both organic and inorganic, temperature, oxygen requirements, hydrogen ion concentration, moisture, liquid or solid mediums, various physical factors such as visible light, infra-red rays, Grenz rays, Roentgen rays (X rays), radium emanations, and perhaps radioactive isotopes. To these must be added genetic factors such as may be observed in cytologic studies and in single spore cultures and which may also be altered



by environment. The additional use of chemical and biologic procedures are important diagnostic criteria.

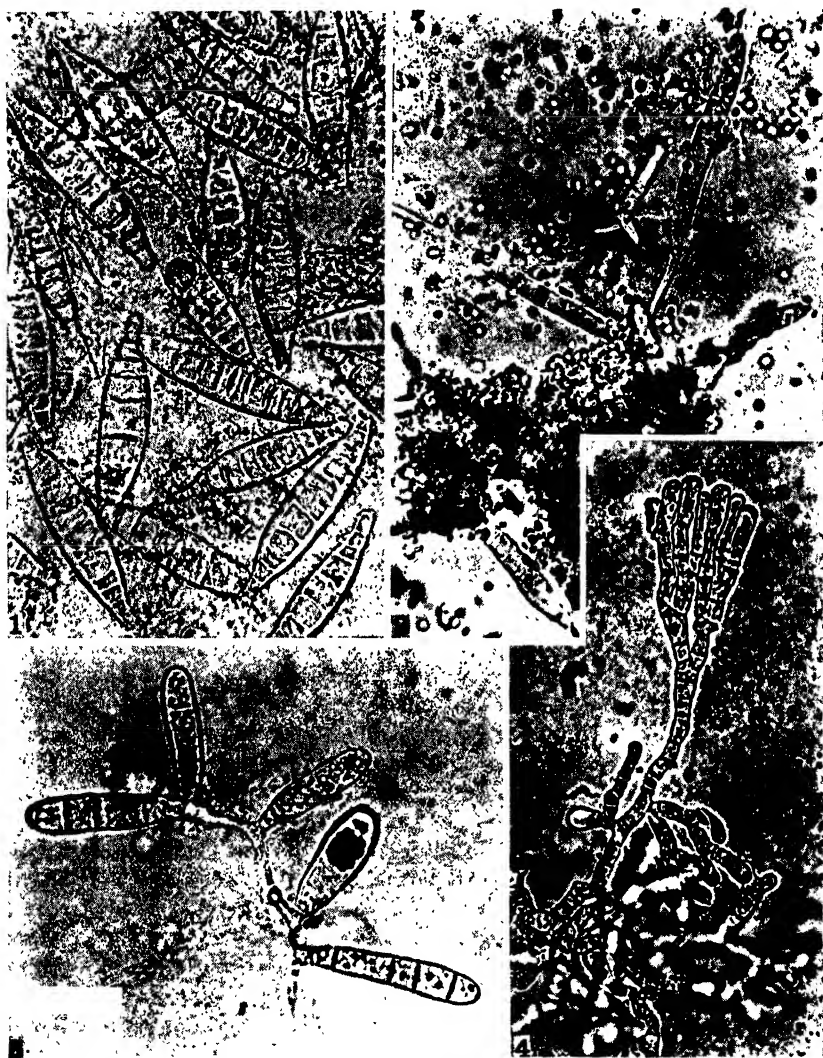


FIGURE 1. Macroconidia or fuseaux of dermatophytes. 1. *Microsporum canis*. 2. *Trichophyton mentagrophytes*. 3. *Epidermophyton floccosum*. 4. *Achorion schoenleinii*.

The nutritional requirements of fungi vary considerably according to the type of fungus, tissue involved, degree of invasiveness, obligatory parasitism, facultative parasitism, and whether the organism exists as a saprophyte within the host or on vegetation outside the host and may, consequently, be air-borne. A medium satisfying the needs of one fungus may have little

or no value for another. Much has been written concerning media and their constituents. The inorganic or mineral constituents of media and their utilization by various fungi have been well investigated chiefly for plant pathogens and nonpathogens. Very little has been done in this regard for human pathogenic fungi, perhaps with the exception of calcium, which was considered to be essential for *Trichophyton interdigitale*.<sup>2</sup> In general, the requirements of fungi for inorganic elements is comparable to those of higher plants. Variations in mineral constituents may alter perceptibly the fungus growth. Organic nutrient requirements, like the inorganic elements, play an important part in the growth essentials of pathogenic fungi. The value of the organic material depends upon its assimilability by the fungi. This in turn depends upon the ability of the fungi to produce or secrete an enzyme which will break down the compound so that the breakdown product can be utilized. To complete the cyclic chain, the production of the required enzymes probably depends upon the pathogenic living conditions of the fungus. The alteration of the organic constituents and the inability of the fungus to utilize them will effect the growth characteristics of the fungus. In addition, there are a number of so-called growth factors, vitamins or specific elements, which play an important part in the development of the sexual phase of the fungus. These factors have important implications in the classification of fungi.

It is generally conceded that the better the environment for growth, the less the probability of the development of the perfect stage. In short, the richer or better the medium, the better the growth of the organism, with the development of vegetative mycelium with few or no reproductive structures. Conversely, the poorer the medium, the better the opportunity for the production of reproductive structures and, consequently, the sexual act and the perfect stage.

No attempt will be made either to describe or to list the numerous preparations in use, past or present. It would be well, however, to mention some of the media in common use. The closest approach to a universal medium is the modified Sabouraud's agar. As originally prepared by Sabouraud, the *milieu d'épreuve* consisted of raw Chanut maltose, granulated Chassaing peptone, agar, and water. Dextrose could be substituted for the maltose. Sabouraud's conservation agar omitted the carbohydrate, so that growth was slow, pleomorphism of the dermatophytes was greatly delayed, and color changes, so important with these organisms, were practically lost in culture. The *milieu d'épreuve*, or test agar, was used exclusively by Sabouraud to produce the giant colonies which served as a basis for identification of the dermatophytes. Unfortunately, this medium contained ingredients which were chemically impure, unstable, and often of such varying composition that giant colonies varied in size, luxuriance of growth, surface markings, and color, from batch to batch. The chief criticisms of this medium have been its instability and the fact that it was almost impossible to develop the giant colonies which Sabouraud illustrated in his "Les Teignes" and which others have used as a basis for comparison in the identification of species. Attempts by investigators in numerous

countries to produce a medium which would duplicate the cultures on Sabouraud's agar resulted in a large number of preparations which failed to attain the objective. The use of honey, as advocated by Sabouraud, likewise introduced a variable form of nutriment with its source of error. In this country, the Pennsylvania medium has been advocated, but the use of crude glucose, although giving good results, again introduces a possible source of error.

In the United States, there is in widespread use a medium also called Sabouraud's agar but which actually may be considered as a completely new substratum. It is made up of peptone, carbohydrate (glucose or maltose, preferably glucose), agar-agar, and water. The ingredients are essentially similar to those of Sabouraud's medium, but in name only, since the American formula consists of standardized, purified products made by the Digestive Ferments Company under the name of Difco. Some laboratories prefer the Pfanstiehl sugars, which are of a better grade. The Difco or Bacto-peptone may be criticized on the ground that it is too near neutral and does not aid in developing the giant colonies illustrated in Sabouraud's "Les Teignes." It has also been condemned by Linder<sup>3</sup> on the ground that "Peptone or peptone and sugar in agar, as used in the past, are not only unbalanced and do not furnish all the elements necessary for the normal growth of the fungi, but, when these substances are supplied, they are in too great concentration." Weidman<sup>4</sup> may, perhaps justifiably, find fault with the fact that Fairchild's peptone and crude glucose are not used.

On the other hand, I have used this modified Sabouraud's agar for somewhat over fifteen years and find that, when used with Difco products in the usual concentration, it is a medium which serves for both rapid isolation and identification of the fungi commonly encountered both in the clinic and in referred patients. To be sure, this medium does not satisfy the needs of all the fungi. Furthermore, it does not bring out all the characteristics desirable and still requires the use of additional media for further study of some fungi. As an all-round medium, however, it serves the purpose well. I am fully aware, too, of some of its other limitations, but, then, to be able to do medical mycological examinations, one needs to have some knowledge of the clinical features of the lesion to be cultured and to use such additional special media as are required. To deal with a pure, standardized medium means that one must set up special criteria for the recognition of the fungi on this medium. The universal adoption of such a medium, whether it be this medium or any other substratum satisfying the general needs of pathogenic fungi, would eliminate much of the dissension among the mycologists who base their identification and classification on a single medium.

Without going into further and greater detail on the very important subject of media, I would like to point out again that the ingredients of a medium determine, in part, the type of growth that will develop. Substrata rich in nutritive value are, as a rule, to be avoided, since for the most part they develop vegetative mycelia without sexual structures. Poor media, on the other hand, are in use to try to induce free sporulation and

the formation of the sexual aspects of the fungus. In most common use are the gypsum blocks, various vegetable plugs, and Gorodkova's agar. On the basis of studies made of *Clenomyces serratus* growing naturally on feathers, it has been suggested that the dermatophytes are related to, or probably members of, the *Gymnoascaceae* of the *Plecoscales*. Because of the similarity of certain structures of this organism, such as spirals and aleurospores, to those seen in the dermatophytes, it has been proposed that these fungi be grown on keratinized substances such as horn, nail, hair, skin, or feathers in order to bring out the relationship. A single microconidium of *Microsporium gypseum* (*M. fulvum*), when grown on horn by Emmons,<sup>5</sup> developed six variants. This unusual condition, unfortunately, could not be attributed with certainty either to the peculiar substratum or to the age of the cultures (four months).

There is evidence that temperature is of definite value in both growth and development of fungi and in the production of sexual characteristics. In general, it has been shown that somewhat lower temperatures are required for the formation of sexual organs than are necessary for vegetative mycelia. Most of the work has been done on nonhuman pathogens, but, for all intents and purposes, it may be assumed that the same applies to human pathogenic fungi. The growth at incubator temperature simulates the vegetative phase of the parasitic state in the human host and, as such, has little value for botanical classification. Consequently, growth at room temperature, approximately 22° C., is much more to be desired, although that may not be the optimum temperature for the specific organism.

Oxygen requirements vary with different fungi. The process of respiration is essentially the same for plants as for animals, but certain fungi, like bacteria, are able to develop in the partial or complete absence of oxygen. The subject of aerobic and anaerobic, or microaerophilic, growth will not be considered except to point out that when the supply of oxygen is limited, as is the case with tight plugs or plugs dipped in paraffin or screw caps for transportation, the growth of the fungus is greatly diminished or stopped. Under such conditions of lowered oxygen tension, sexual reproductive processes may be inhibited. On the other hand, the organism *Actinomyces israeli* (*A. bovis*) will grow only in a microaerophilic state. This, of course, brings up the question as to whether one should classify a fungus purely on its oxygen requirements, such as *Actinomyces* or *Nocardia*, *Nocardia* being an aerobic form. It is my feeling that, unless the organisms show sufficient morphological or other variations, they should not be classified in separate genera purely on the basis of oxygen requirements. The value of partial anaerobiosis has been well demonstrated in the case of *Coccidioides immitis*, where the development of endosporulation *in vitro* takes place only under partial anaerobic conditions.<sup>6</sup>

Hydrogen ion concentration (pH) likewise may play a role in classification. For general purposes, the hydrogen ion concentration in cultural work may be disregarded, since the range of growth of the fungus is fairly wide. For critical work, however, the pH of the medium may have an important bearing. Talice<sup>7</sup> studied a number of pathogenic fungi grown on

three different media with varying hydrogen ion concentrations, and indicated the pH at which minimum, optimum, and maximum growth took place. He noted frequently gross variations in the form and color of the colonies, especially in the highly tinted species and, oftentimes, variations in the microscopic characters of the fungi. Microscopically, he noted a predominance of one form of vegetation according to the pH value.

Moisture is usually a requisite in mycological investigation and classification. Most organisms, especially yeasts and yeastlike forms, require moisture both for growth and for the germination of spores. Liquid media are used for both the study of spore germination and the production of certain chemicals and vitamins which may be elaborated in a medium as a result of the growth processes of special fungi. Since, among the Fungi Imperfecti, classification depends to some extent on the sporulation of the fungus, moisture is of great importance. At the present time, we employ both liquid and solid media. The solid substrata used today, however, are chiefly of the nature of colloidal gels, with the exception of specialized solid media. These colloidal gels require moisture and as such serve the purpose, except that germination of spores or free spore formation is usually much slower unless stimulated by temperature changes. Such media often produce an abundance of vegetative mycelia but are of less value for the formation of reproductive or sexual organs. Occasionally, too, various spores require alternate wetting and drying for maximum germination. Although wet media are the rule in mycology, very often old, dried-out cultures develop specialized structures, such as sclerotia, which may help considerably in arriving at a classification of the fungus.

Various physical factors have been employed in mycology, but chiefly to determine their lethal effect on fungi. These include visible light, infra-red rays, ultraviolet rays, Grenz or infraroentgen rays, Roentgen rays (X rays), and radium emanations. Visible light has been shown to have a definite morphogenic influence on certain fungi, as well as to stimulate the development of reproductive structures. Pathogenic fungi, in general, are very little affected by visible light. Most of the work done on fungi has been with the rays of shorter wave lengths. Smith,<sup>8</sup> in 1936, induced variants in fungi experimentally by using suitable exposures of ultraviolet radiations. Hollaender and Emmons<sup>9</sup> (also Emmons and Hollaender<sup>10</sup>), working with the effect of measured amounts of monochromatic ultraviolet radiation on spores of the dermatophyte *Trichophyton mentagrophytes*, found that wave lengths of 2280 to 2950 Ångströms affected these spores. The lethal effect and the secondary mutant formation occurred with wave lengths of 2537 and 3650 Ångströms, with a maximum of  $100 \times 10^{-4}$  ergs per spore. (With measured amounts and increased time of exposure, the amount of mutations decreased.) The effect of the sublethal radiation manifested itself by the production of small, slow-growing colonies. The spores that survived the lethal dose produced colonies which resulted in the mutants. These mutants differed from normal controls in their size, form, rate of growth, spore production, and color. Unfortunately, genetic studies could not be

made because of the lack of the perfect stage, the sexual organs. Spores of old cultures, however, were able to produce similar mutants.

Muskatblit and Ouspensky,<sup>11</sup> using Grenz rays on hairs affected with *Microsporium audouini*, *M. canis*, *Trichophyton crateriforme*, *T. violaceum*, and *Achorion (Trichophyton) schoenleinii*, found doses up to 50,000 roentgens ineffective on the gross or microscopic morphology of these fungi. Fungi exposed to Roentgen or X rays showed changes. Some of the colonies developed color changes, slow rate of growth, and small amount of mycelial development with colony sectoring. Many of the fungi were considered to be saltants, since the effects of the radiation wore off in subsequent subcultures. Others, however, persisted as mutants. Sartory, Sartory, and Meyer<sup>12</sup> exposed *Aspergillus fumigatus* to 3–7.2 millicuries of radium emanations and were able to induce morphologic changes in the organism. Large-celled oidia, thick-walled spores, and large pseudosporangia were produced on media that were practically salt free. When cultures were exposed to higher doses, hard, fusiform sclerotia containing perithecia were found in the subsurface mycelium. It is evident that physical factors may induce morphologic and, perhaps, genetic changes in fungi in sufficient amount to have an effect on the classification of these organisms. No doubt additional work will be done with these agents, to which may be added the recently developed radioactive isotopes, which, at present, are an unknown quantity in many fields of biologic endeavor.

One branch of biology that has been sadly neglected in medical mycologic classification and in its general application to pathogenic fungi is cytology and its genetic implications. This may be attributed chiefly to the consideration that most pathogenic fungi belong to the Fungi Imperfecti, and genetic studies cannot be made because of the lack of suitable material, such as the ascospores of *Neurospora* which have been so fruitful for so many investigators. In spite of the apparent lack of sexual forms in most pathogenic fungi, however, much can still be accomplished in determining generic or even species relationships and thus helping to arrive at a logical classification. Monospore cultures such as were made by Emmons for *Microsporium gypseum* (*M. fulvum*) are a step in this direction. Certainly the cultivation of single spores of all types may bring interesting and worthwhile results into the present disturbed system of classification.

Of great significance, too, are the cytological investigations of pathogenic fungi. This phase has been passed over too lightly by mycologists. This has been so, perhaps, because of the technical procedure that is involved in making such a study. In the Fungi Imperfecti, cytological investigations would amply repay the worker with the vast amount of information that could be obtained. In the absence of essential criteria, which make classification difficult, the cytological data should be of great benefit. If we were to consider pathogenic fungi in general and the Imperfecti in particular to be degenerate forms of the sexually perfect groups of fungi, then it is quite possible that light would be shed on the reproductive organs or processes by investigating the various cellular forms of the fungi under varying con-

ditions of growth. Cytological investigations should be highly recommended to the young enthusiasts of mycology.

In addition, there are other procedures which are essential if pathogenic fungi are to be classified properly. These include various biochemical studies such as fermentation, especially useful in the case of yeastlike organisms, assimilation of carbon and of nitrogen, gelatin liquefaction, hydrolysis of starch, indol production, and others, some of which may or may not have practical value. In addition, biological and serobiological reactions have also been applied in mycological classification, and these include agglutination determinations, immunological reactions, and others.

What has been presented so far may be summarized briefly as representing the tools of the mycologist for the determination and classification of pathogenic fungi. How wisely these tools are used and how well the results are interpreted will determine the accuracy of the diagnosis and classification.

To keep the botanist from straying too far from a logical and sane system of classification, a binomial nomenclature has become established and is being maintained by the taxonomists, who are guided by a set of International Rules of Botanical Nomenclature. These have been reprinted by Dodge<sup>13</sup> with examples pertaining to pathogenic fungi. Many pleas have already been made for strict adherence to and complete adoption of these rules. Needless to say, those of us who are familiar with these rules seek to do all we can to respect them. Inadvertently, however, most of us have at one time or another been guilty of either neglect, forgetfulness, or unconcern. Much of the confusion existing in our present system of classification has arisen from the simple fact that these rules have been disregarded and personal interpretations have been substituted. These rules are as pertinent to pathogenic fungi as they are to nonpathogens. The importance of adhering to the use of the Latin diagnosis in the description of new genera or species has already been stressed. The omission of the Latin diagnosis for newly described fungi makes the new name invalid. While this is important from the standpoint of taxonomists, there are other rules of great significance which have been disregarded.

The avoidance and violation of the principle of priority has been responsible for a share of the chaos in the classification of pathogenic fungi. This rule refers to the maintaining of the oldest and first binomial given to a group or species. Unfortunately, this infraction could be excused on the ground that it was unintentional or that the early descriptions were brief, incomplete, and poorly illustrated, or that they appeared in a journal which was not readily accessible. Since, in many instances, the original publications are not seen, the investigators depend upon the quotations present in the papers of others. Interpretations based on such descriptions varied according to the group studying them, and, as a consequence, there have arisen groups or schools who follow the interpretations or concepts of certain leaders. As a result of such conceptions or misconceptions, it is not unusual to find that the name applied to the organism by the original worker may be used by present workers to refer to an entirely different fungus. I be-

lieve the term *gypseum* is one which has been responsible for this type of confusion. An outstanding example of the misuse of terms is that concerned with the agent or agents of moniliasis. The term *Monilia*, which has been applied to the organism of moniliasis and which is falling into disrepute, was adopted as the cause of this disease (FIGURE 2). The present-day description of this genus and its usage as related to pathogenic fungi is totally different from the original concept. The creation of the genus *Syringospora* by Quinquaud in 1868, based on the *Oidium albicans* of Robin, is undoubtedly the first valid and legal name for the organism of moniliasis and as such should replace the various genera being used for the organism in question. As a result of the misinterpretation of the older legalized terminology, there developed a type of sentimentality in classification with

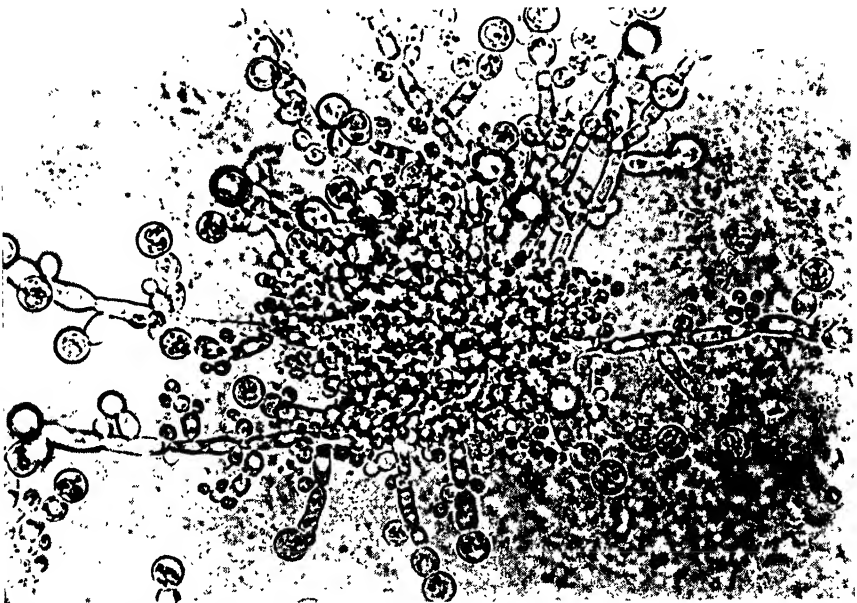


FIGURE 2. *Syringospora albicans* grown on corn-meal agar. Note large, characteristic chlamydospores.

each group of workers adhering to the ideas and ideals of the exponents of these groups. Consequently, we find that many adhere to the genus *Candida* of Berkhout, published in 1923, which in its present-day usage refers to *Syringospora* Quinquaud, and in that sense is invalid. In fact, the whole group of yeastlike organisms is badly in need of monographic studies. Several fine papers have already been published with regard to the species of *Syringospora*, but these have been referred to the genera *Mycotorula*, *Candida*, or *Monilia*.<sup>14</sup> Many of the species have been either reduced to synonymy or eliminated, but there are still other species and genera which need classification through monographic study.

Another outcome of the incomplete descriptions or poor illustrations of the earlier workers has been the development of two widely separated



groups, the "lumpers" and the "splitters." The lumpers tend to decrease the number of genera and species to a few, whereas the splitters favor the creation of new genera and species based on the interpretation of new or different characters. Simplification of classification is, of course, highly commendable and it makes the remembering of names fairly easy, especially for the none too botanically-minded medical men. On the other hand, we must avoid oversimplification, for, in the end, this will defeat the purpose of taxonomy. The splitters may be criticized for the creation of too many genera and species, although some may be justifiable. A compromise group is the ideal, and this should be the goal of the taxonomists.

A recent example of this is illustrated by the organisms causing chromomycosis or chromoblastomycosis. On the basis of the various characters produced in culture, the fungi were provisionally separated into several genera, *Phialoconidiophora*, *Phialophora*, *Hormodendroides*, *Botrytoides*, and *Hormodendrum*, and placed in the order *Phialophorae* at a time when there was a state of confusion existing in the classification of these pathogens (FIGURE 3).<sup>15</sup> Because of the various morphological characteristics in culture, the fungi had been variously classified as *Acrotheca*, *Gomphinarina*, *Trichosporium*, *Hormodendrum*, and then *Fonsecaea*. In contrast, one of the recent publications would place all the organisms with their assorted characteristics in the one genus, *Phialophora*, on the basis of finding the cupuliform spore bearers in all the strains.<sup>16</sup> I believe that this group of fungi needs further study for final disposition.

In some instances, the choice of a name has led to the discarding of the valid one in favor of one which was more descriptive, according to the likes of the investigator. What we call a fungus is not so important as knowing what that fungus does and where in the classification it belongs. Even if we are not in favor of the name given these microorganisms, let us strive to attain at least some semblance of order by retaining the legitimate name given by the investigator. In this regard, the rules state clearly that "a name or epithet must not be rejected, changed, or modified merely because it is badly chosen, or disagreeable, or because another is preferable or better known." There have been numerous instances of this violation in the classification of pathogenic fungi.

The rule stating that only one name may be given to an organism regardless of how many pleomorphic forms or life cycles it may have does much to alleviate the situation concerning the dermatophytes and other fungi with a variable life cycle. It would seem, therefore, that at best the classification of most pathogenic fungi is probably tentative, subject to the eventual finding of the perfect stage. At that time, all previous names, no matter how well established or contested, will have to be discarded in favor of the binomial or at least generic classification with which the perfect stage is associated. An example of this may be cited in the case of *Monosporium apiospermum*, the perfect stage of which is reputedly *Allescheria boydii*.<sup>17</sup>

The present system of classification is based on the work of Saccardo, as shown in his "Sylloge Fungorum." An attempt was made to bring to-

gether, in orderly fashion, organisms of like morphology or with essentially similar characters, based on a probable phylogenetic sequence dependent upon the ontogeny of the related fungi. In this method of classification



FIGURE 3. Organisms of chromomycosis. 1. *Phialophora verrucosa*. 2. *Hormodendrum* sp. 3. *Hormodendroides pedrosi*. 4. *Phialoconidiophora guggenheimia*. 5. *Botrytoides monophora*.

then, an attempt is made to follow a natural system of evolution, with the most complex form of life being the latest and the simplest form being the oldest. The division of the fungi into classes, orders, families, genera, and species is essentially an indication of relationship based on the evolutionary

processes. Although the tendency is to consider it as following natural lines, much of it may be considered to be artificial until more concrete evidence is presented. Most pathogenic fungi do not present a significant amount of evidence on which to base a phyletic sequence, except as relates to individual groups. Thus, before we can assume a definite system of classification, such as exists with other fungi, we must diligently apply ourselves to the investigation of these fungi. Since the present classification of fungi is man-made, we should not expect it to be perfect; and there are those who may accordingly disagree with the form and substance of such a classification. Any attempt to alter the system for the better should, therefore, be given careful and encouraging consideration.

Pathogenic fungi for the most part have been isolated, studied, and classified by medical men who patterned their classification chiefly along clinical lines. The particular fungus was related to the clinical features of the disease, both macro- and microscopically. The monumental work of Sabouraud and of the earlier workers, who helped lay the cornerstone for the classification of the dermatophytes and of medical mycology in general, followed very much along natural lines (FIGURE 4). The value of their work undoubtedly is great, so great that, from a clinical standpoint and from the point of view of the dermatologist, it has not been surpassed and is still in use. This, of course, brings up the question of whether the classification of pathogenic fungi should be made to fit the needs of the medical man or of the pure mycologist. It is desirable to have a classification which would facilitate diagnosis and, from an economic and practical viewpoint, help both the medical man and the patient. The difficulty with such a classification, however, lies in the fact that the diversity of lesions that a fungus may produce may be so great as to be confusing. Consequently, it is felt by many that a botanical classification developed along natural lines may be the only possible solution to this problem. Accordingly, Dodge, basing his work on the morphology of the fungus in both the lesion and in culture mediums, developed a compromise classification. On the other hand, Emons, adhering to the morphologic characters of the fungus in culture alone, classified the fungi purely on a mycologic basis, with the result that the natural lines of both the clinical and mycologic classifications show crossing at some points. In spite of the confusion that exists as a result of such classification, I feel that such difficulties will be ironed out in due course.

It is generally conceded that a botanical classification, based on the characters of the fungi (cultural, biologic, and microscopic), is the logical and scientific method for differentiating these organisms. In their pathogenic state, however, fungi present certain criteria which help to place them in certain groups. To this end, we should consider the arbitrary clinical classification of mycoses and their causative agents, based on anatomic distribution and also on their ability to invade tissue. Briefly, mycoses may be considered clinically as belonging to one of several groups: (1) superficial mycoses affecting only the *stratum corneum* of the skin or the hair, never becoming invasive or systemic; (2) dermatomycoses involving and invading the superficial and deep layers of the skin and also the hair and nails

# TABLEAU SYNTHETIQUE DES DERMATOPHYTES

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(1) Les espèces les plus importantes ont leur nom en italique.

(2) A côté du *Trichophyton fumatum*, et, en tout cas, parmi les Trichophyton endothrix il faudra bientôt faire place à deux nouvelles espèces, isolées à Venise, dans le service de M. le Prof. Fiocco, par le Dr Minassian. L'une: *Trichophyton inflatum* à culture cerebriforme, poudreuse et craquelée, présente en son centre une boursouffure blanchâtre, difforme, caractéristique sur milieu d'épreuve. L'autre: *Trichophyton spongoides*, présente une boursouffure centrale analogue mais de couleur bistre, neutre, non poudreuse et semblable à une éponge brune déposée sur une aréole poudreuse, craquelée.

FIGURE 4. Sabouraud's classification of the dermatophytes. (Reproduced from *Les Teignes*, p. 281. 1910. Masson et Cie. Paris).

(there is no evidence of systemic involvement); and (3) mycoses usually primarily involving the skin or mucous membranes or both, systemic invasion being either primary or secondary and systemic infections, chiefly primary, with occasional or rare secondary cutaneous involvement.

Fungi producing disease in the first group are, fortunately, few in number and present well-established forms which make them easily recognizable. Although there is some disagreement as to the naming of these fungi, it is only a matter of time and careful study of these organisms before definite taxonomic differentiation will be made. In the second group, the dermatophytes, careful study of the fungi in the parasitic state will reveal sufficient differences in morphology to allow for allocation at least to genera. In many instances, however, because of age and various environmental factors, the physiology of the fungi has become so altered that morphologic characteristics merge one group into another. In these instances, cultivation on artificial media becomes a matter of necessity not only for genus but also for species determination. Although the dermatophytes have received perhaps the greatest amount of attention, there are still several points that need elucidation. Here, a good knowledge of the clinical aspects of the disease plus a background of general mycology, as well as medical mycology, is of tremendous importance in classification.

Organisms producing disease of the internal organs or a combination of cutaneous, mucous membrane and systemic lesions are, in general, recognized with relative ease. There are, however, several criteria which play an important part and may easily confuse the not too well trained. Fungi seen in tissue, blood, pus, or cerebrospinal fluid, at body temperature, develop morphologic structures which remain fairly constant in the parasitized host. The organisms in this state present the parasitic phase of the life cycle. As such, they may be classified as to groups and type of disease, but give no structures which can be classified botanically. Whole blood, serum, and tissue extracts are utilized in artificial media and maintained at body temperature in the incubator to continue this phase of the fungus life cycle. Although this serves the purpose described, the specific classification of the organism cannot be accomplished by this means. A frequent error in the classification of fungi in the parasitic state is in referring to all budding organisms as "blastomycetes." The term "blastomycete" brings to mind the misnamed organism of blastomycosis, *Blastomyces dermatitidis*. It is readily understandable, therefore, that such means of classification are not only misleading but also confusing. Several fungi show budding forms in the parasitic phase such as *Syringospora* (*Monilia*, *Candida*), *Cryptococcus*, *Histoplasma* (which may have both small and large forms), *Sporotrichum*, and *Paracoccidioides* in addition to the fungus of Gilchrist's disease (FIGURE 5). Obviously, such confusion can be dispersed by growing the organism on a suitable medium which will bring out specific characteristics. More significant, however, would be to discard the term "*Blastomyces*," which rapidly is assuming the role of a *nomen ambiguum*.

Much progress has been made in the classification of pathogenic fungi. Much more will have to be done to develop a readily understandable, work-

able, and biologic system for these organisms. For the botanically-minded mycologist, the pure mycological classification is the only solution, and for the medically-minded mycologist a system involving clinical aspects would

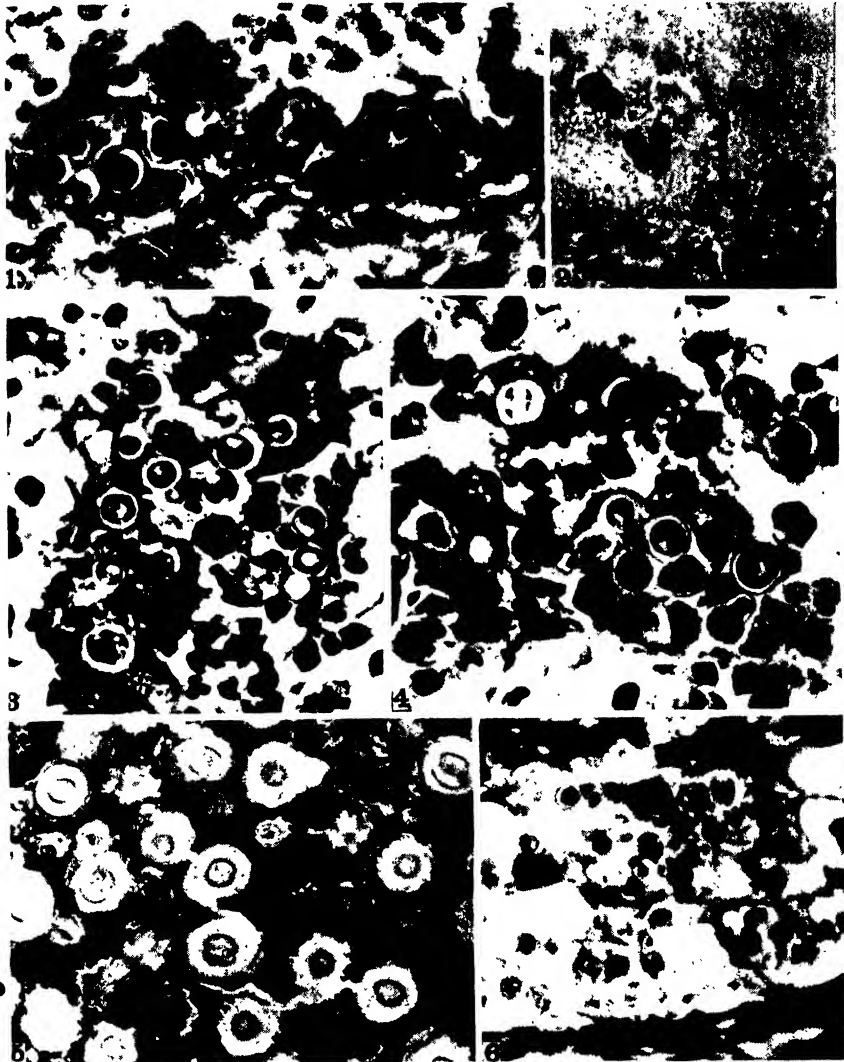


FIGURE 5. Budding, yeastlike pathogenic fungi in tissue. 1. *Zymonema dermatitidis* in the adrenal. 2. *Syringospora albicans* in nail scraping. 3. *Paracoccidioides brasiliensis* in lymph node. 4. *Histoplasma capsulatum* in the prostate. 5. *Cryptococcus neoformans* in the brain. 6. *Sporotrichum schenckii*, experimental inoculation in the mouse testis.

help considerably to facilitate clinical diagnosis in a language readily understandable by the clinician. To help alleviate the confusion existing in the botanical classification, the adherence to a set of guiding rules such as pro-

posed by the Committee on International Rules of Botanical Nomenclature is essential for uniformity of classification throughout the civilized world. I feel that an international subcommittee devoting itself to pathogenic fungi exclusively and to which new organisms with their new designations could be submitted for final disposition would eliminate future confusion. At the same time, I feel that some provision should be made for a clinical classification which would benefit the medical man and yet not hamper the botanical classification of pathogenic fungi. This perhaps is a big order, but I firmly believe that the one may complement the other and yet each be valuable individually.

From the foregoing discussion one may reasonably conclude that the status of the classification of pathogenic fungi is as yet unstable and is replete with confusion and chaos. To be sure, we have not attained the perfection in classification that is to be desired, but the future is not dark. As long as we have workers interested in the classification of pathogenic fungi, and trained mycologists who are willing to adopt the dynamic methods of classification, then confusion will be dispelled and order will reign. Medical mycology is still a young science, and where there is youth there is hope.

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# FUTURE DEVELOPMENTS IN MYCOLOGICAL INVESTIGATIVE METHODS

By Norman F. Conant

*Department of Bacteriology, Duke University School of Medicine, Durham, North Carolina*

Although a fungus was the first recognized infectious agent of man and animal, relatively little is known about fungus diseases in comparison with the accumulated knowledge concerning the epidemiology, immunology, immunochemistry, and specific therapy of bacterial and virus infections. The lack of such information about fungus infections has been the result of methods of study which stressed the clinical aspects of the diseases and the taxonomy of the fungi almost to the exclusion of fundamental studies of the infectious agents and the disease processes which they caused. At the present time, however, there are few disagreements concerning the pathogenicity and the taxonomy of those fungi which produce superficial or systemic mycoses. The dermatophytes are no longer represented by a vast, complicated, and confused group of organisms. Rather, they have been reduced to a small number of easily recognized and identified fungi. The systemic fungi, likewise, have evolved through a period of recognition, classification, and confusion, and now are also known to be represented by a small number of easily recognized and identified fungi. It is hoped, therefore, that investigative methods may now be directed more fully towards gaining a better understanding of the physiological, chemical, immunological, and epidemiological aspects of the human pathogenic fungi and the diseases they produce.

A trend toward the use of such mycological investigative methods has already been forecast by recent studies which have made use of these tools. For example, growth requirements of the fungi are no longer studied by changing brands of peptone and types of sugar in the media. Robbins and Ma (1942, 1944, 1945) have investigated the nutritional requirements of *Trichophyton discoides*, *Rhodotorula* sp., and *Trichophyton mentagrophytes* by their behavior on a synthetic basal agar medium or synthetic basal solution to which various vitamins and other growth-promoting substances could be added. Hazen (1947) has reported preliminary nutritional studies on *Microsporium audouini* and has shown that growth factors present in yeast extract stimulate conidial production and colony formation. A further study of the exact requirements for a better growth of *M. audouini* is to be reported later. Since microscopic morphology and gross cultural characteristics are used to identify the pathogenic fungi, the stabilization of such characters by growth on a synthetic medium, to which can be added known substances, would allow duplication and a more stable taxonomy. The value of a method of study that would lead to such a stabilization is, of course, readily apparent.

Physiological investigations of the human pathogenic fungi also have made use of methods that provide basic quantitative information concerning the activity of the organisms. Bernheim (1942), Nickerson (1946), and



Nickerson and Chadwick (1946) have indicated the value of respiration studies (oxygen consumption) as a method of measuring the *quantitative* effect of toxic substances on living, pathogenic fungi. Investigations of this type might also indicate the enzyme system or systems involved in the oxidation of various substances when drugs are added to the preparations and oxidation is hindered. This type of information, obtained by such studies, could lead to definite points of attack against the pathogenic fungi, since it not only gives quantitative data about the toxicity of drugs but also indicates their mode of action.

Chemical investigations of the human pathogenic fungi are needed to yield valuable information concerning the metabolic products which they produce and to which allergy or immunity is established in the infected human or animal. Trichophytin, coccidioidin, blastomycin, and, lately, histoplasmin, are sterile filtrates of a liquid medium in which the respective fungi have been grown for long periods of time. Such materials, when injected intradermally, give delayed tuberculinlike reactions which denote present or past infection by the homologous fungi.

Purified products of some of these materials have been obtained and comparable studies with the "crude" filtrates have been made. "Crude" trichophytin and its purified products have been used in studies relating to infection, immunity, and sensitization caused by the dermatophytes. Reviews and personal investigations contained in reports by Sulzberger (1932), Jadassohn, Schaaf, and Wohler (1937), DeLamater and Benham (1938), and DeLamater (1941) have shown that infection by this group of fungi causes sensitization that can be demonstrated by skin tests, and that these fungi contain common or group antigens. Infection by one member of the group, therefore, may cause sensitization that can be demonstrated by a skin test, not only to the homologous extract but also to an extract of other dermatophytes. Extracts of fungi not belonging to this group were found not to cross-react.

There have been recent reports, however, which indicate that some of the dermatophytes produce antigens or reagents which sensitize the skin to products produced by markedly different fungi. For example, penicillin allergy in patients who have never received penicillin is thought to be established by previous infection of the skin by species of dermatophytes. Peck and Hewitt (1945) showed that some dermatophytes produce a penicillinlike substance, and Cormia and Lewis (1946) showed a correlation between penicillin sensitivity and sensitivity to superficial fungus infections. Peck and Siegal (1947) attribute penicillin sensitivity to an infection of the skin by dermatophytes which produce the penicillinlike substance which, in turn, creates the sensitivity that is elicited when penicillin is used therapeutically. These later studies show, therefore, that widely different fungi do contain common antigenic substances that may prepare patients for later allergic manifestations. These facts have great clinical significance, and more should be known about the types of products produced by the dermatophytes, since these fungi cause widespread infection in the population.

Although the sensitizing antigens contained in trichophytin have been studied extensively, not much is known about the same types of materials that may be present in extracts of fungi which cause systemic infections. *Coccidioides immitis* is known to produce a substance or substances in broth cultures which cause a specific reaction in patients and experimentally infected animals. This material, coccidioidin, may also be used as the antigen in complement fixation tests and in precipitin reactions. Hirsch and Benson (1927) showed filtrates of cultures of *C. immitis* to give specific skin tests, and that heating at 80° C. for 30 minutes did not destroy its activity. Also, prolonged electrodialysis caused the formation of white floccules which, when dissolved in distilled water with N/100 NaOH, gave positive tests comparable to those evoked by the original coccidioidin. Hirsch and D'Andrea (1927) were able to recover a "specific soluble substance" from coccidioidin by alcohol precipitation. More recently, Hassid, Baker, and McCready (1943) isolated a polysaccharide from coccidioidin, which gave positive skin tests to 0.00001 mg. injected intradermally and gave a precipitate in serum and pleural fluid in a dilution of 1:640,000. These few reports indicate the nature of the specific substance in culture filtrates of *C. immitis*. The "crude" filtrate, coccidioidin, has been used, however, as a specific skin-testing material in epidemiological studies to determine the rate of infection in the population and to determine the endemic foci of coccidioidomycosis in this country. The clinical significance of an established sensitivity to *C. immitis* should be investigated.

In North American blastomycosis (Gilchrist's disease), an investigation of the significance of an established sensitivity to *Blastomyces dermatitidis* has been reported by Martin and Smith (1939). They have shown that patients sensitive to a skin test dose of a standardized heat-killed *Blastomyces* vaccine could not be given iodides safely without previous desensitization. In such patients, infection spread rapidly when iodides were given in the presence of sensitivity to the organism. Peck, Martin, and Hauser (1940) isolated polysaccharides from the yeast phase of *B. dermatitidis* and found that this type of material gave positive skin tests and also could be used for desensitization. The allergic state of the patient in this disease, therefore, must be established before treatment can be instituted. Not only should the allergic state of a patient infected with *B. dermatitidis* be established before rational treatment can be given, but the immunologic status of the patient should also be known. Martin and Jones (1941) were able to treat successfully, with immune rabbit serum, a patient with a negative skin test, a negative complement fixation test, but a positive Foshay type of test (positive skin test to immune rabbit serum). Such a patient was thought to have excess antigen produced by the fungus, which masked the skin test and inhibited antibody formation. By injecting immune rabbit serum, the patient's immunologic status was reversed and response to treatment was immediate. A similar type of immunologic finding has also been reported by Hiatt and Martin (1946) in a patient with pulmonary moniliasis. Rapid clearing of the lungs followed treatment with anti-albicans rabbit serum in a patient showing a positive Foshay type of skin

test to immune serum, but in whom there was a negative skin test to vaccine and a negative agglutination test. In this patient, it was thought that excess antigen produced by *Candida albicans* masked the skin test and inhibited antibody formation. In these two instances, therefore, an understanding of the patient's immune response was necessary for successful treatment. Information concerning the significance of sensitivity and immune responses in other types of systemic fungus infections may prove equally as important.

While the allergic and immune status of an individual patient should be studied with all of the available techniques, it is also of great importance to establish the rate of infection of a given fungus disease in the population for a better understanding of that disease. Epidemiologic studies of two systemic fungus infections (coccidioidomycosis and histoplasmosis) have changed the concept of these diseases. Both were thought to be highly fatal infections until skin test materials were developed that could detect minimal or past infections by eliciting a positive skin test, comparable to and interpreted in the same way as the tuberculin test. Gifford (1936) and Dickson (1937) were the first to demonstrate that a primary benign type of infection was caused by *Coccidioides immitis*. Inhabitants in the known endemic areas of the disease have shown a high incidence of infection, as determined by positive coccidioidin tests. The secondary malignant progressive phase of coccidioidomycosis (coccidioidal granuloma) was found to occur infrequently and to be more prevalent in the dark-skinned races.

Until recently, histoplasmosis has been considered a highly fatal infection. Christie and Peterson (1945), Palmer (1945 and 1946), and Furcolow, High, and Allen (1946), however, have shown that, in certain parts of the country, there is a high correlation between pulmonary calcification in nontuberculin reactors and positive skin tests to histoplasmin. If this skin-testing material proves to be specific (Emmons, Olson, and Eldridge, 1945; Howell, 1947), it will have shown that *Histoplasma capsulatum* also causes a primary benign type of infection and that the highly fatal form of the disease represents only a not too frequent secondary malignant type of infection.

It is hoped that the future development of mycological methods will stress these physiological, chemical, immunological, and epidemiological approaches and will yield a better understanding of the human pathogenic fungi. Much has already been accomplished, and the methods of study have been indicated. Such a program will need the help of adequately trained personnel. The personnel necessary for effective work would include a mycologist, a physiologist, an immunochemist, and a pathologist. This group should be in contact with clinical facilities that would provide cases for study and should be in contact with clinicians who would help select the problems to be investigated.

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# SUPERFICIAL DERMATOMYCOSES CAUSED BY *TRICHOPHYTONS*, *MICROSPORUMS*, AND *EPIDERMOPHYTONS*

By Fred D. Weidman

*Department of Dermatology and Syphilology, School of Medicine,  
University of Pennsylvania, Philadelphia, Pa.*

If one were to consult a standard text on dermatology,<sup>1</sup> he would find such a long list of superficial fungus diseases caused by *Trichophyton*s, *Microsporums*, and *Epidermophyton*s that he would realize at once the impossibility of making a comprehensive presentation, even in a monograph like this. I have decided, therefore, to steer a course which will be panoramic, hoping to inform the general scientist and general pathologist, even at the expense of the dermatologist and skilled mycologist. In so doing, I will follow the avenue of the general pathologist and attempt to trace the natural history of the diseases in the premises.

Accordingly, this panorama, first, will confine itself to dermatomycoses that are superficial (ringworm and favus). Second, it will deal with the pathology of the group as a whole. In connection therewith, certain features will be pointed out that either have been underemphasized or have some special application to medical biology at large. To a large extent, these special points have a bearing upon diagnosis and treatment, which are so important to the dermatologist.

*Habitat of the Fungi on Vegetation and Animals.* I am not aware of authenticated evidence that any of the three genera of fungi cited in the title live and grow upon vegetation in nature. Members of all of them, however, frequently parasitize the skin of lower animals: cats, dogs, horses, cattle, sheep, monkeys, and others.<sup>2</sup> In short, the diseases that they produce can be contracted from lower animals, but, at the same time, it is thoroughly established that most of them are contracted from man to man. Details in respect to animals can be found in the recent volume by Hull.<sup>3</sup> Kadisch<sup>4</sup> has reviewed the subject in general.

*Transmission.* This is a story that is too extensive and complex to elaborate in detail here. Suffice it to point out that one is dealing with fungous cells which live upon the surface of an animal or of man, and which need only come in contact with the appropriate (receptive) soil on the surface of a human being. Moreover, after being shed from the animal (including man), the spores can lie dormant and viable in the environment for as much as three months in the case of *Trichophyton mentagrophytes*<sup>6, 7</sup> and twelve months for *Microsporum felineum*.<sup>5</sup>

*Predisposing Factors for Infection.* Considering the ubiquity of superficial fungus disease, and the readiness with which the infectious material can be dispersed into the environment, it is clear that many human skins are immune to infection. Otherwise, practically all human beings would become infected. There is ample evidence of the close and continued contact of infected skins with healthy ones. Many wives are free from dermatophytosis of the toes (athlete's foot), whereas their husbands' toes are

notably diseased. Again, in a family of ten, only one or two members will have favus—a favus, moreover, that is of several years' duration. The remaining eight must be immune. Natural immunity accounts for many of these cases, but certain investigations<sup>8</sup> make it appear that a high pH of the cutaneous surface plays a role in preventing infection. Acquired immunity is indicated in the skin at a pre-existing patch of favus which has been found immune against experimental reinoculation for up to three months.<sup>9</sup> Singularly, the endocrine system plays a role in tinea capitis. Witness the fact that the infection can disappear spontaneously at puberty and can be made to disappear under treatment by stilbesterol. In short, there is an outstandingly large factor of "the soil" in connection with that successful invasion of the skin which we call infection.

Moisture, especially from sweating, is conducive to infection, especially in intertriginous places.

*The Tissue Reaction.* This is the "morbid anatomy" of the general pathologist. The range of tissue reaction is variable, *but is confined to the skin*. Singularly, the disease does not extend to the internal organs. Even when fungus spores are forcibly rubbed into denuded skin and reach internal organs, they survive only a few hours.<sup>10</sup> It is assumed that the fungus perishes for lack of oxygen.

In respect to the tissue reaction of the skin, the simplest is that in which only the epidermis participates. That is, an inflammatory infiltration does not develop in the corium. This simple reaction is not represented in any of the diseases caused by *Trichophyton*, *Microsporum*, and *Epidermophyton*. It is represented in tinea versicolor. Here, little more than a moderate hyperplasia of epidermal cells and hyperpigmentation take place. The hyperplasia results in excessive keratinization, and the end result is a macule which is more or less brown, slightly scaly, and scarcely congested. Incidentally, the disease, not uncommonly, has a predilection for the orifices of the hair follicles, thus resulting in a stippled patterning. On the face of it, this would speak for the presence of nutritive materials which originated in the pilosebaceous apparatus; but the matter could be much more complex than this. In any event, there is a hint here toward the investigation of factors which might favor the growth of this fungus species.

All three genera are concerned in the next higher level of tissue reaction, such as is exemplified in ringworm of the scalp (tinea circinata) and dermatophytosis. Here, it is definitely inflammatory. The story is the conventional one in respect to the connective tissue reaction, namely, congestion and exudation. The congestion is reflected in the redness observed clinically. The exudation may mount to such proportions as to produce a vesicle or even a bulla. In one form of ringworm of the scalp (kerion), it is so severe as to result in suppuration. The epidermis participates in the form of a cellular hyperplasia (which the dermatologist denominates "acanthosis"), together with edema. The fungus is confined to the epidermis. In the case of vesicles and bullae, it is most abundant on the underside of the ceiling of the vesicle, and a practical suggestion therefrom is that the dermatologist should point toward this region when he is examining mate-

rial microscopically for fungus for purposes of diagnosis. That is, the bottom of the ceiling should be arranged "upward" on the microscopic slide. Thereby, the fungus will not be obscured to the extent that it would be otherwise.

Depending upon the individual case, then, the lesion either will be simply congested and hyperkeratotic (scaly), as in the simpler cases, or will have vesiculation and bulla formation added, as in severe ones. Secondary infection accounts for the presence of pus in both vesicles and bullae.

Secondary effects are well established in the form of ascending lymphangitis and cellulitis. In addition, certain claims have been made that dermatophytosis is responsible for thromboangiitis obliterans, but it is too soon as yet to accept this theory as proven.<sup>11</sup>

*Mycotic Folliculitis (Ringworm and Favus).* Here, the infection extends more deeply below the surface, i.e., into the deepest parts of the hair follicle (tinea capitis, tinea barbae, and favus). With the exception of favus, the fungus still invades only the epidermis, in principle, if one recalls that the sheath of the follicle and its contained hair shaft are but invaginations of surface epithelium. The fungus gains entry at the follicular orifice, after which it proceeds downward through the hair shaft. It induces degeneration of the shaft, which results in its loosening from the hair papilla. The hair falls out or is broken off, resulting in baldness. Fortunately, the papilla itself is not destroyed, except in favus, and after the disease has regressed, it resumes the formation of hair shafts. The baldness is only temporary. In infections with *Microsporum felineum* the reaction is so intense that suppuration ensues, i.e., suppurative folliculitis. This is the "kerion" of the dermatologist.

There is one fungus species, *Trichophyton schoenleinii*, which extends beyond the hair follicle into the connective tissue roundabout. It is the cause of the disease favus. It destroys the hair papilla, from which the hair shaft grows, with the result that permanent baldness results. The damage that is produced in the connective tissues results in scarring. This *Trichophyton* is the only member of the three genera under discussion which produces permanent damage to the skin.

*Immunity.* In spite of the extremely superficial diseases produced by these three fungus genera, their products diffuse sufficiently into the corium to act as antigens. This results in allergic tissue reactions in more or less distant parts of the skin, which are known as dermatophytids. Naturally, such lesions do not contain fungus substance. The type of reaction (lesion) is so highly varied that it cannot be described in detail here. Suffice it to say that the lesions are of such varied type and distribution as to imitate those of other dermatoses so closely that even the most skilled dermatologist is commonly baffled by them.<sup>12</sup> As to the usefulness of immunologic reactions for purposes of diagnosis, it is enough to say that they are practically nil, whether cutaneous or serologic.

*Laboratory Diagnosis.* Although the principles of technique conform to those of bacteriology, the methods are radically different and much simpler. This is so, principally, because both fungus cells and colonies are so much

larger and characteristic that resort need not be had as frequently to biologic tests. Commonly, the morphology in the test tube or under the microscope suffices. For details, see the text by Lewis and Hopper.<sup>1</sup> Incidentally, ultraviolet light (Wood's light), played upon a fungus colony, induces differences in color which have diagnostic value, but the usefulness of this method is impaired because much skill is entailed in the reading of the colors. The assistance of a skilled mycologist is required here. Such a light, however, has come into general use clinically. When directed upon a lesion, the fungus-infected materials fluoresce spectacularly. The species of fungus is not indicated in such examinations. Only the fact that the disease is mycotic is demonstrated.

*Treatment.* As in the past, fungicidal chemicals are the main reliance today, together with X-ray epilation in the case of ringworm of the scalp.<sup>13</sup> One notable advance has been made by discovering fungicidal value in certain fatty acids. These have the advantage that they do not induce untoward inflammatory reactions. Hopkins and his co-workers<sup>14</sup> investigated them in respect to dermatophytosis, employing volunteers at Army establishments during World War II, and fixed upon propionates and undecylenates. Peck and Russ<sup>15</sup> prefer propionate-caprylic mixtures. The entire subject, including the requirements for fungicide testing, has been reviewed recently.<sup>16</sup>

In the treatment of ringworm of the scalp (tinea capitis), it has been discovered recently that fungicidal applications suffice to cure infections by *Microsporum felineum*, whereas infections by *Microsporum audouini* remain resistant and require X-ray treatment. This has led to increased demands upon the laboratory for the identification of the fungus species in the case.

In any event, many of the ringworm infections are resistant to cure, even under the best circumstances. This has led to praiseworthy attempts in other directions than the stereotyped fungicidal chemicals. Thus, Chambers and Weidman<sup>17</sup> discovered a fungistatic strain of *Bacillus subtilis* in normal toes which inhibited a pathogenic fungus of ringworm of the toes. That is, antibiotic therapy was essayed. Lewis<sup>18</sup> has continued such studies, but the results have not been sufficiently proven.

Poth<sup>19</sup> secured good results in the treatment of ringworm of the scalp by the administration of stilbesterol by mouth, but results have not been sufficiently uniform in other hands to establish this method. There is a logical basis for this form of treatment, because it is an established fact that tinea capitis disappears spontaneously at puberty and is almost unknown after that time.

Dolce and Nickerson<sup>20</sup> employed zinc chloride locally on the basis that it might inhibit respiration of the fungus. Although they failed, it is nevertheless true, as they pointed out, that it is the avenues directed against the biology of the fungus that should be followed in future investigations. Only in this way can one expect to emerge from the welter of stereotyped, empirically selected chemical fungicides and uncover substances that are epoch-making in therapy.



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# CHROMOBLASTOMYCOSIS\*

By Arturo L. Carrión

School of Tropical Medicine, San Juan, Puerto Rico

## Clinical Aspects

**Definition.** Chromoblastomycosis is a chronic, infectious, apparently noncontagious skin disease confined most frequently to one of the lower extremities and characterized clinically by the formation of nodular, verrucous, or tumorlike lesions. The infection may be caused by any of several species of dematiaceous fungi.

**Synonymy.** The term "chromoblastomycosis," first used by Terra *et al.*<sup>2</sup> in 1922, is not only a long word, but a misnomer, falsely ascribing the disease to a *Blastomyces* and conveying the erroneous impression of an extraordinary color element in the clinical picture. Notwithstanding this, we believe that, for the present, this name should be preserved. It has been extensively used in the literature and it expresses the frequent resemblance of the disease to true blastomycosis. Furthermore, the prefix "chromo," though misleading in regard to the clinical picture, is descriptive of the etiologic fungus.

The following synonyms have been used or suggested for this disease: *Blastomycose negra* (Pedroso<sup>3</sup>); *figueira* (Rudolph<sup>4</sup>); *dermatite verrucosa por Phialophora verrucosa* (Pedroso and Gomes<sup>5</sup>); *formigueiro* (Gomes<sup>6</sup>); chromomycosis (Moore and Almeida<sup>7</sup>); *dermatite verrucosa cromomicótica* (Redaelli<sup>8</sup>); *dermatitis verrucosa blastomicótica* (Boggino<sup>9</sup>); Pedroso's disease, Fonseca's disease, or Gomes' disease (Weidman and Rosenthal<sup>10</sup>); Pedroso and Carrión's disease (Barros-Barreto<sup>11</sup>). Some of the synonyms just mentioned are almost as long or longer than chromoblastomycosis and are still misnomers; others might be criticized for bearing personal names. If the term "chromoblastomycosis" were to be changed at all, it should be done at an international congress, where the subject could be thoroughly discussed and settled by general agreement.

**History.** In 1911, Pedroso, of São Paulo, Brazil, noted the presence of large, dark-brown to yellowish, spherical bodies in a biopsy from a patient with nodular and ulcerated skin lesions of the foot and leg (Pedroso and Gomes<sup>5</sup>). He suspected a mycotic infection and was able to isolate from the lesions a dark-colored fungus. The disease became known as *blastomycose negra* in Pedroso's laboratory (Pedroso<sup>3</sup>), but the study of the presumptive causative agent was postponed and the discovery was not reported until 1920.<sup>5</sup>

In 1914, Rudolph<sup>4</sup> published his observations on a skin disease popularly known as *figueira* in Minas Geraes and Goyaz. The clinical description and mycologic findings given by Rudolph in his report clearly indicate that he was dealing with the same disease observed by Pedroso in São Paulo three years before.

\* In the preparation of this paper, the author has partly transcribed and partly abstracted the information contained in a chapter on chromoblastomycosis previously prepared for "Biology of Pathogenic Fungi" (Annales Cryptogamici et Phytopathologici) by Carrión and Silva.<sup>1</sup> Some additional information has also been included.

In 1915, Medlar<sup>12</sup> and Lane,<sup>13</sup> in the United States, published their observations on "a cutaneous infection caused by a new fungus . . ." in a patient from Boston. The lesions of this patient were of blastomycetic type and contained numerous spherical, pigmented, parasitic cells. The etiologic fungus was carefully studied and named *Phialophora verrucosa*. From the descriptions of Medlar and Lane, it was apparent that the disease in the Boston patient was identical in nature with that previously observed in Brazilian patients by Pedroso<sup>5</sup> and Rudolph.<sup>4</sup>

In 1922, Brumpt<sup>14</sup> established that the organism causing the infection in Pedroso's original case was not *Phialophora verrucosa*, but a new species, which he named *Hormodendrum Pedrosoi*. Subsequent studies on the morphology of the latter fungus have led to one of the most interesting chapters in the history of chromoblastomycosis. In 1923, Fonseca and Leao<sup>15</sup> described for this fungus a second method of sporulation which corresponds closely with the genus *Acrotheca*. In 1935, it was found in our laboratories<sup>16</sup> that the species *Pedrosoi* possesses still another method of sporulation by which the conidia are produced in phialides, thus establishing its relationship to a third form genus, namely, *Phialophora Medlar*, 1915.<sup>17</sup> The simultaneous occurrence of three methods of sporulation in one and the same organism and the various proportions in which these methods may be represented in different isolates of the parasite led to the description of the species under different specific and generic names. The literature on the subject became extremely confusing. However, after years of painstaking work in our laboratories,<sup>18,19</sup> the synonymy of these names became firmly established, and the binomial *Fonsecaea Pedrosoi*, proposed by Negroni,<sup>20</sup> of Argentina, has been accepted as the most convenient name until the perfect form of the fungus becomes known.

In 1935, we described, in a Puerto Rican case, a third etiologic agent for chromoblastomycosis. The Puerto Rican fungus reveals the three methods of sporulation characteristic of the genus *Fonsecaea*, but it possesses sufficiently distinct characteristics to warrant its registration as a new species which bears the name *Fonsecaea compactum*.<sup>21,22</sup>

According to the above observations, it is evident that chromoblastomycosis, like mycetoma, may be caused by many different fungus parasites. In addition to the three etiologic fungi just reviewed, a few others which appear to be legitimate species have been recently described. Although the latter species are not generally recognized, they are worthy of consideration and will be discussed later under "Mycologic Aspects."

*Incidence and Geographic Distribution.* The existence of chromoblastomycosis has been established in North, South, and Central America, the West Indies, Europe, Africa, the East Indies, Japan, and Australia, leaving continental Asia as the only part of the world as yet without reported cases. Among the infections classed as chromoblastomycosis, there are 159 in which the diagnosis appears to have been made on a sound basis, and this number might be accepted as an approximate index of the recognized cases up to the year 1947. These cases are distributed as follows: Cuba, 43; Brazil, 41; Puerto Rico, 15\*; South Africa, 12; United States, 9;

\* Among the 15 Puerto Rican cases, there are 8 which have not been officially published yet.

Venezuela, 9; Russia, 5; Costa Rica, 4; Dutch East Indies, 4; Japan, 3; Algiers, Argentina, and Australia, each, 2; and Rhodesia, Dominican Republic, Guatemala, Canal Zone, Mexico, Canada, Paraguay, and Uruguay, one case each. By plotting these cases on the map, it will be found that 127, or 80 per cent, occurred in tropical or subtropical regions, and only 32, or 20 per cent, in temperate zones. According to these observations, chromoblastomycosis might be considered as a cosmopolitan disease, but its distribution would be predominantly tropical.

*Symptomatology.* The disease usually affects one of the lower extremities. It begins as a small papule or warty growth, which may develop anywhere on the extremity, but is located, as a rule, on some part of the foot, whence the infection spreads upward through the gradual development of satellite lesions. The course of the pathologic process is slow, and the



FIGURE 1. A Puerto Rican case of chromoblastomycosis of fifteen years' duration. The lesions occurred in great numbers, especially toward the distal portion of the extremity, and they were conspicuously varied in morphology.

clinical history often reveals that the infection has existed for ten or more years at the time of examination.

In a typical well-advanced case of chromoblastomycosis, the foot and leg are generally swollen and somewhat elephantiasic in appearance (FIGURE 1). The lesions occur in great numbers, especially toward the distal portion of the extremity, and they are conspicuously varied in morphology. For the purpose of description, it is convenient to class them in five different types, namely, the nodular, the tumorous, the verrucous, the plaque, and the cicatricial.

The nodular type includes the youngest and smallest elements in the clinical picture and consists of moderately elevated, fairly soft, dull-pink to violaceous growths, the surface of which may be smooth, verrucous, or scaly.

Through further development, many nodules are gradually transformed into lesions of the second, or tumorous, type. This type is represented by much larger and more prominent, distinctly papillomatous, sometimes

lobulated, tumorlike masses, partly or wholly covered with dirty gray epidermal débris, crusts, and horny particles. On the foot and lower leg, where the pathologic process tends to be most exuberant, the tumor masses often reach enormous dimensions, taking on the characteristic appearance of cauliflowers.

In the third, or verrucous, type of lesion, hyperkeratosis is the outstanding feature; the efflorescences are warty in appearance and may resemble *verruca vulgaris*. Growths of the verrucous type are frequently encountered along the borders of the foot.

The plaque type is the least common of the lesions of chromoblastomycosis. It consists of fairly flat, slightly elevated, variously sized and shaped areas of infiltration. They are reddish to violaceous in color, superficially scaly, and some of them show exaggeration of the lines of cleavage. The development of small, papillomatous vegetations, or larger nodules, within a plaque is sometimes observed and may lead to great variations in the morphology. When present in the clinical picture, the plaques are generally found on the higher portions of the extremity and never on the lower leg or foot.

Finally, the cicatricial type of lesion is represented by growths that enlarge by peripheral extension, while healing takes place at the center with the production of sclerotic or atrophic scarring. Cicatricial lesions may cover more or less extensive areas and are usually annular, arciform, or serpiginous in contour.

The lesions of chromoblastomycosis develop slowly but progressively, and, in the course of time, a few or many of them may coalesce to form extensive and often bizarre aggregates. The infected tissues are easily traumatized and bleed readily. When pressed with the fingers, many of the lesions discharge a whitish, caseous material and sometimes fluid pus at one or more points. Secondary bacterial infections and ulceration frequently complicate the clinical picture and are mostly responsible for the foul-smelling character of the eruption, a feature that is usually perceptible at a distance from the patient. Subjectively, pruritus may be an important symptom, and some patients complain of pain. In advanced cases, there is partial or total incapacity for work.

The deeper tissues are not usually involved. The lymphatic glands draining the diseased focus may participate in the process, but this is not the rule. However, adenitis due to bacterial complications is not infrequent. Metastases through the blood stream appear to be extremely rare, but there is no question that they can be produced (Carrión and Koppisch<sup>23</sup>). Finally, no systemic symptoms have yet been recorded from the infection.

It should be emphasized that the dermatologic picture just given is a general representation of the symptomatology in well-developed, typical cases of chromoblastomycosis of the lower extremities. Although location in one of the lower extremities is the rule, the disease may affect the skin in almost any other part of the body, either as aberrant lesions produced during the course of a limb infection, or as the original site of attack. Involvement of the upper extremities ranks second in order of frequency, the initial lesion usually being located somewhere on the hand or wrist, although it may ap-

pear in any other segment of the limb. Infection of the face has been recorded in three instances, while the neck and trunk have been involved only once each and in different cases.

*Clinical Types.* In chromoblastomycosis, as in any other chronic, infectious dermatosis, the clinical type of the infection in individual patients may vary in accordance with the duration of illness, the degree of virulence of the infecting parasite, the nature of the host reaction, and the location of the pathologic process. Different clinical types of the disease have been reported (Carrión,<sup>21</sup> Pardo-Castello<sup>24</sup>) according to the predominance of one of the types of lesion above described. The possible correlation of clinical types with one or another of the specific parasites producing the disease has been suggested by various workers, but our present knowledge is too limited to warrant final conclusions on this phase of the subject.

*Histopathology.* Chromoblastomycosis falls into the group of infectious granulomas. The pathologic process affects both the cutis and the epidermis, and it develops toward the surface with little or no tendency to involve the deeper structures. Infection is apparently extended to adjacent regions by autoinoculation through the epidermis, or by way of the superficial lymphatics (Carrión and Koppisch,<sup>23</sup> Mériin,<sup>25</sup> Edson de Almeida,<sup>26</sup> Briceño-Iragorri<sup>27</sup>). Metastases through the blood stream are very rare, but may be produced (Carrión and Koppisch<sup>23</sup>).

When the lesions are examined microscopically, the epidermis is generally thickened and often folded to fit the underlying papillomatous elevations (FIGURE 2). The thickening is due largely to hyperplasia of the *stratum Malpighii*, which not only is broader than normal but shows irregular growths penetrating more or less deeply into the cutis (FIGURES 2 and 3). These growths may be sufficiently pronounced to resemble an epithelioma of the prickle cell type. The *stratum corneum* shows marked hyperkeratosis and is often distorted. Polymorphonuclear leucocytes are sometimes noted infiltrating the epidermal layers and, not infrequently, they form miliary, abscesslike accumulations.

In the cutis, the pathologic reaction is essentially granulomatous, with a varied cellular infiltrate consisting of lymphocytes, plasma cells, large mononuclear leucocytes, polymorphonuclear leucocytes, eosinophiles, Russell's fuchsin bodies, epithelioid cells, and occasional giant cells of both the Langhan's and foreign body types. The infiltrate may be focal or diffuse. In many places, it is distinctly tuberculoid (FIGURE 4). Miliary abscesses are frequently observed, but widespread necrosis, softening, and free supuration are not so common. The affected tissues show a constant tendency toward fibrosis as a natural defense mechanism to wall off the infective process. This tendency becomes especially noticeable in the older foci of infection, where the major part of the lesions often consists of fibrous tissue.

The most significant character in the pathologic picture is undoubtedly the presence of the infecting fungus (FIGURE 4). This may be seen as rounded, occasionally crescent-shaped (FIGURE 5), often septate bodies measuring about ten microns in diameter, with fairly thick and dark cell walls and a coarsely granular protoplasm possessing a pigment which has

been variously described as ochre, olivaceous, yellowish-green, and dark chestnut. The parasitic cells may occur singly or in groups. In the cutis they are variously located within giant cells, free in the tissues, and, not infrequently, in the center of microabscesses, but they may be found also within epithelial pearls and microabscesses in the epidermal layers. Evidence of germination is often noted in the *stratum corneum*.

*Etiologic Factors.* Chromoblastomycosis occurs most frequently during the period of active adult life. Among 109 authentic cases of the disease



FIGURE 2. Histopathologic section of a lesion in chromoblastomycosis. The epidermis is thickened and folded to fit the underlying papillomatous elevations. In the cutis, the reaction is essentially granulomatous, with a varied cellular infiltrate.

in which the age was recorded, 77 (nearly 71 per cent) lay between the ages of 20 and 50 years. The extreme ages at which infection took place were 3 (Tschernjowski<sup>28</sup>) and 76 (Takahashi<sup>29</sup>) years, respectively.

Chromoblastomycosis is decidedly more common among males. A review of 138 authentic cases in which the sex was registered showed that 132 were males, a proportion of 96 per cent.

There seems to be no race immunity. However, in a collection of 124 clinical histories containing data regarding the race of the patients, it was

found that 74 cases, or 60 per cent, were Caucasians, and 38 cases, or 30 per cent, were Negroes. Other races reported in the remaining 10 per cent included: the Mongolian (6 cases), the Malayan (2 cases), the Hindu (2 cases), a Mexican mestizo, and a Jamaican.

A large majority of the victims of chromoblastomycosis have been farm laborers working barefooted in the fields at the time of infection. In a study of 73 patients whose histories include the occupation, it was found that 60, or 82 per cent, were engaged in this type of work, while 3 others were farmers



FIGURE 3. Histopathologic section of a lesion in chromoblastomycosis. The thickening of the epidermis is due largely to hyperplasia of the *stratum Malpighii*, which is not only broader than normal but shows irregular growths penetrating into the cutis.

who were also engaged in other occupations (carpentry, mining, and nursing).

The specific causative fungi of chromoblastomycosis will be discussed under "Mycologic Aspects" (*q.v.*).

*Treatment.* When the lesions are small and discrete, it may be possible to eradicate completely the infection by surgical excision or electrocoagulation. On the contrary, when the infection is advanced and the lesions numerous, extensive, and confluent, these measures may be impracticable



*Fonsecaea compactum*, *Phialophora verrucosa*, *Torula poikilospora*, and two *Hormodendrum* species described respectively by Simson<sup>32</sup> and O'Daly.<sup>33</sup> The second group consists of four organisms which produce primarily soft, dark, moist colonies undergoing what appears to be a yeast-like phase in the course of their development. As the cultures age, the thallus becomes partly filamentous, but the growth as a whole retains its moist appearance. Only two of the organisms of this group have been published: *Hormiscium dermatitidis*, by Kano,<sup>34</sup> and a "black *Candida*-like . . . species," described by Berger.<sup>35</sup> Of the two remaining fungi of this group, one was isolated by C. Bonne in the East Indies, and the other was obtained by us in Puerto Rico. The descriptions of these two fungi are not yet available.

*Fonsecaea Pedrosoi*. Authorship: Brumpt, 1922<sup>14</sup>; Negroni, 1936, *comb. nov.*<sup>20</sup>; Carrión, 1940, *emend.*<sup>18</sup>

*Synonymy.* *Hormodendrum Pedrosoi* (Brumpt<sup>14</sup>); *Phialophora verrucosa* (Pedroso and Gomes<sup>5</sup>); *Acrothecha Pedrosoi* (Fonseca and Leao<sup>15</sup>); *Hormodendrum algeriensis* (Montpellier and Catanei<sup>36</sup>); *Acrothecha Pedrosiana* (Bonne<sup>37</sup>); *Trichosporium Pedrosianum* (Ota<sup>38</sup>); *Acrothecha verrucosa* (Tschernjowski<sup>38</sup>); *Trichosporium Pedrosoi* (Langeron<sup>39</sup>); *Hormodendrum rossicum*<sup>3</sup> (Meriin<sup>40</sup>); *Cladosporium algeriensis* (Vuillemin<sup>41</sup>); *Gomphinarina Pedrosoi* (Dodge<sup>42</sup>); *Hormodendroides Pedrosoi* (Moore and Almeida<sup>43</sup>); *Phialophora macrospora* (Moore and Almeida<sup>43</sup>); *Botryloides monophora* (Moore and Almeida<sup>43</sup>); *Phialoconidiophora Guggenheimia* (Moore and Almeida<sup>43</sup>); *Hormodendrum japonicum*\* (Takahashi<sup>29</sup>); *Hormodendrum Negroni*† (Pereira<sup>45</sup>); *Carrionia Pedrosoi* (Briceno-Irgaorri<sup>46</sup>); *Hormodendrum chaquense*† (Mazza and Niño<sup>47</sup>); *Phialophora Pedrosoi* (Binford *et al.*<sup>48</sup>).

*Geographic Distribution.* *Fonsecaea Pedrosoi* has never been found in nature outside of the human body, but the widespread distribution of its victims has established its ubiquity. It is the most common etiologic agent of chromoblastomycosis. Among 90 legitimately classified organisms isolated from the disease throughout the world, 76, or 84 per cent, belong to this species. The geographic distribution of the fungus is conspicuously higher in the warmer climates. This is evident from the fact that 63, or 83 per cent, of the 76 isolates just mentioned, were obtained from patients who contracted the infection in tropical or subtropical regions.

The continental distribution of *Fonsecaea Pedrosoi* has been as follows: North America, 5 isolates, (4 from the United States and 1 from Mexico); Central America, 4 isolates, (3 from the Panama Canal Zone† and 1 from Guatemala); South America, 25 isolates, (19 from Brazil, 4 from Venezuela, and 2 from Argentina); West Indies, 31 isolates, (18 from Cuba, 11 from Puerto Rico,§ and 2 from the Dominican Republic||); Europe, 4 isolates,

\* The fungus described under this name was not available for comparative studies in our laboratories, but the original description of the organism, together with the published illustrations, is strong evidence of its identity with *Fonsecaea Pedrosoi*.

† Authentic cultures of the so-called *Hormodendrum Negroni* and *Hormodendrum chaquense*, respectively, were kindly sent to us by Dr. Juan A. Mackinnon, of Uruguay. We agree with Azulay<sup>44</sup> that these two fungi are identical with *Fonsecaea Pedrosoi*.

‡ Of the three isolates from the Canal Zone, only one has appeared in the literature (Snow *et al.*<sup>49</sup>); the other two were studied in our laboratories due to the courtesy of Drs. E. S. Wedding and C. Calero, respectively.

§ Only four of the Puerto Rican isolates have appeared in the literature (Carrión and Koppisch<sup>20</sup>; Carrión<sup>40</sup>).

|| Of the two isolates from the Dominican Republic, only one has appeared in the literature, as far as we know (Carrión and Pimentel-Imbert<sup>51</sup>). The other was studied by the author due to the courtesy of M. F. Pimentel-Imbert.

(all of them from Russia); Africa, 4 isolates, (1 from Algiers and 3 from the Union of South Africa); Asia, 1 isolate from Japan; and the East Indies, 2 isolates, (1 from Java and 1 from Sumatra).

**Morphology in Pathologic Tissue.** Since chromoblastomycosis in human beings is essentially a skin disease, the infecting fungus is to be found almost exclusively in cutaneous lesions. Here it may be recognized in both the dermis and epidermis as characteristic, usually spherical, occasionally crescent-shaped, brownish-yellow bodies, so-called "sclerotic cells," measuring about 10 microns in diameter (FIGURES 4 and 5). These bodies may occur either singly or in clumps, rarely in short chains. They are found within giant cells, free in the tissues, in the center of microabscesses, or enclosed within epithelial pearls in the Malpighian and horny layers of the epidermis.

The fungus elements possess a dark, fairly thick cell wall, part of which is occasionally swollen, bulging toward the interior of the cell. The wall is sometimes covered with a crusty layer of refractile material (FIGURE 6a). The protoplasm is granular, contains refractile inclusions, and possesses a

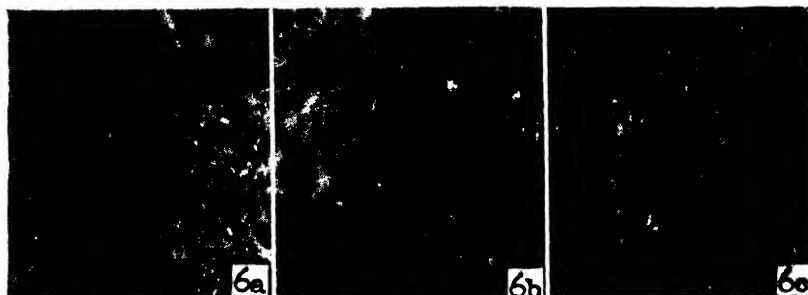


FIGURE 6. *Fonsecaea Pedrosii* in the infected epidermal scales: (a) isolated fungus cell covered with a crusty layer of refractile material; (b) fungus cells in different stages of division; (c) fungus cell showing internal septation in two planes, and also germination.

conspicuous natural pigment which has been described as ochre, olivaceous, yellowish-green, and chestnut brown. The nucleus is not apparent in fresh or stained preparations. Germination of the parasitic cells is frequently observed in the *stratum corneum* of the epidermis (FIGURE 6c). In the infected tissue, multiplication of the fungus takes place by fission, different stages of the process being noticeable in both the dermis and epidermis (FIGURE 6b).

**Gross Morphology in Culture.** On Sabouraud's *milieu d'épreuve* and on 4 per cent dextrose agar, after the fourth week and at room temperature, cultures resemble flattened cones, measuring about 5.5 cm. in diameter (FIGURE 7); center of culture is elevated about 8 mm. above medium, sometimes forming mammillary prominence; rest of culture gently slopes, often radially folded; the aerial mycelium forms dark gray, greenish, olivaceous gray, or dark brownish, feltlike network. On Czapek's agar, colonies are poorly developed; mycelium is mostly submerged, gray to olivaceous, forming at border, arborescent outgrowths; central zone shows shallow layer of aerial hyphae.

**Microscopic Morphology in Culture.** The microscopic characters are usu-

ally more conspicuous after the third week of growth. The vegetative hyphae are long, straight or undulated, 1.25 to 3 microns in diameter, septate, branching, cell walls thick and dark; protoplasm olivaceous, granular, with refractile droplets.

The sporulation is of three different types, namely, the *Hormodendrum*, the *Fonsecaea*, and the *Phialophora* types. These methods of sporulation may be occasionally combined in the same spore head. The *Hormodendrum* type (FIGURES 8i, 8j, and 8k) may be more or less abundant. It is generally more conspicuous on Czapek's agar cultures, and is sometimes predominating, sometimes obsolete, according to the individual isolate. The fertile

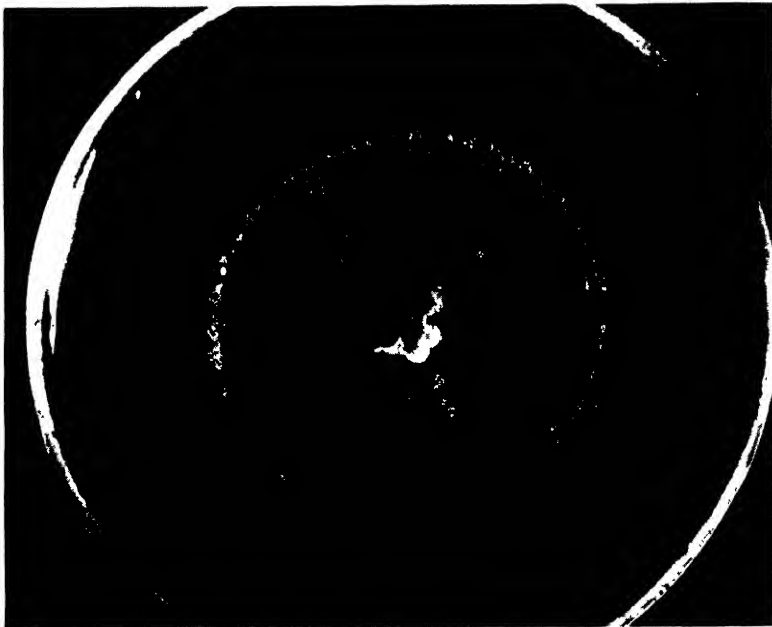


FIGURE 7. *Fonsecaea Pedrosoi*: culture four weeks old developed at room temperature on Sabouraud's "milieu d'épreuve."

branches are erect or ascending, with terminal cell or conidiophore sometimes darker, having at the tip several tiny, truncate, conical prominences to which the spores are attached. The conidia are borne in chains, usually short, which tend to branch by multiple budding at the distal pole of each successive conidia, resulting in the formation of complicated spore heads. The conidia are unicellular, ovoid, often elongated, those at the base of the chain being frequently shield-shaped and occasionally bicellular. They measure 3 to 5 by 1.5 to 3 microns (basal elements 2.5 to 3.5 by 7 to 10 microns), are olivaceous in color, have smooth, thick, and dark walls and moderately to poorly developed disjunctors. The *Fonsecaea* type of sporulation (FIGURES 8a to 8h) is more or less abundant, usually more conspicuous on corn-meal agar cultures, and sometimes predominating, sometimes ob-

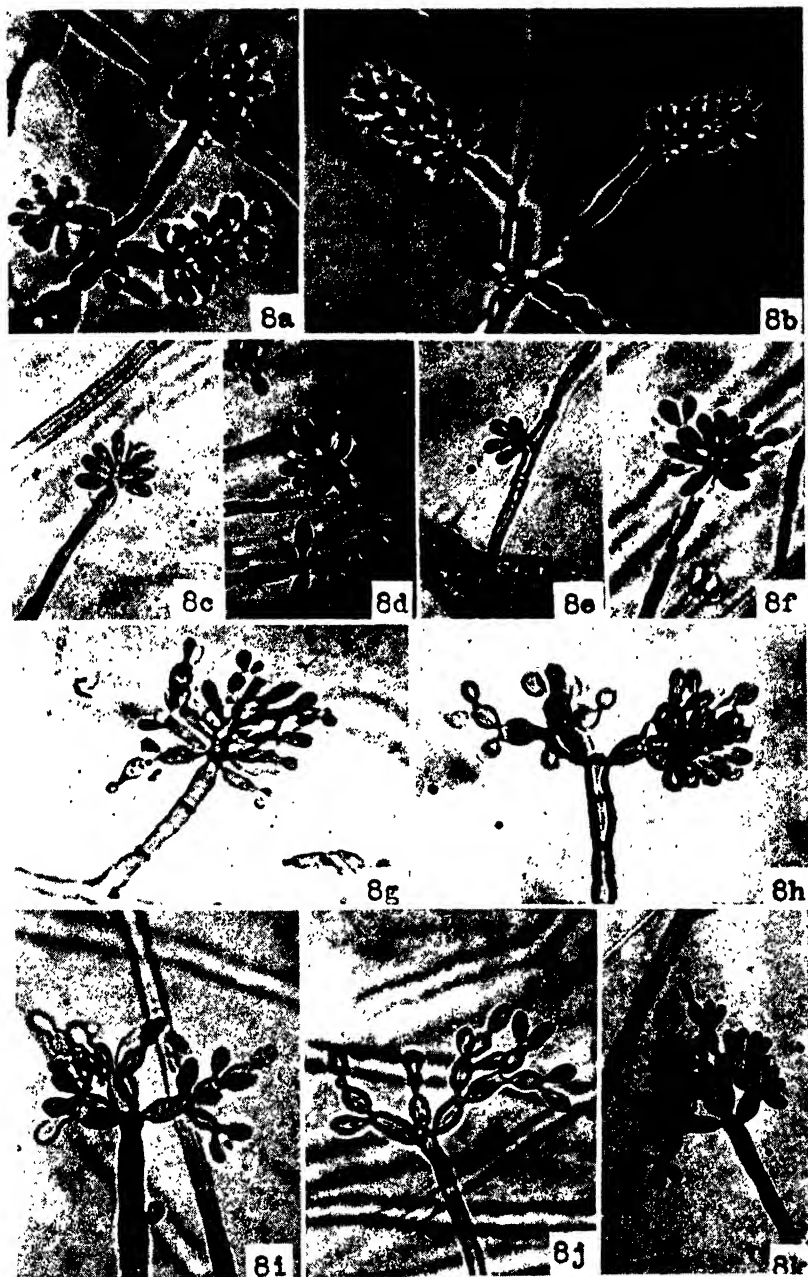


FIGURE 8. *Fonsecaea Pedrosi*, microscopic morphology: (a, b, and c) *Fonsecaea* type of sporulation, here resembling *Acrotheca*; (d and e) *Fonsecaea* type of sporulation, lateral conidiophores with conidia; (f and g) *Fonsecaea* type of sporulation showing chain formation of *Hormodendrum* type; (h) *Fonsecaea* type of sporulation developed on *Hormodendrum* spore in *Hormodendrum* head; (i, j, and k) *Hormodendrum* type of sporulation showing short chains, profuse branching, and multiple successive budding of spores.

solete, according to individual isolate. The conidiophores are short or long, straight or irregular, exceptionally branched, consisting of one, sometimes more, articles disposed terminally, laterally, or intercalarily, often derived from spore element in *Hormodendrum* head (FIGURE 8h). The surface of the conidiophore is wholly or partly verrucous, due to the presence of tiny, truncate, conical prominences to which the spores are attached; its pigmentation often is darker than vegetative mycelium. The conidia are sometimes single (not catenate), forming clusters which may be indistinguishable, when terminal, from those of *Acrotheca* (FIGURES 8a, 8b, and 8c), some clus-

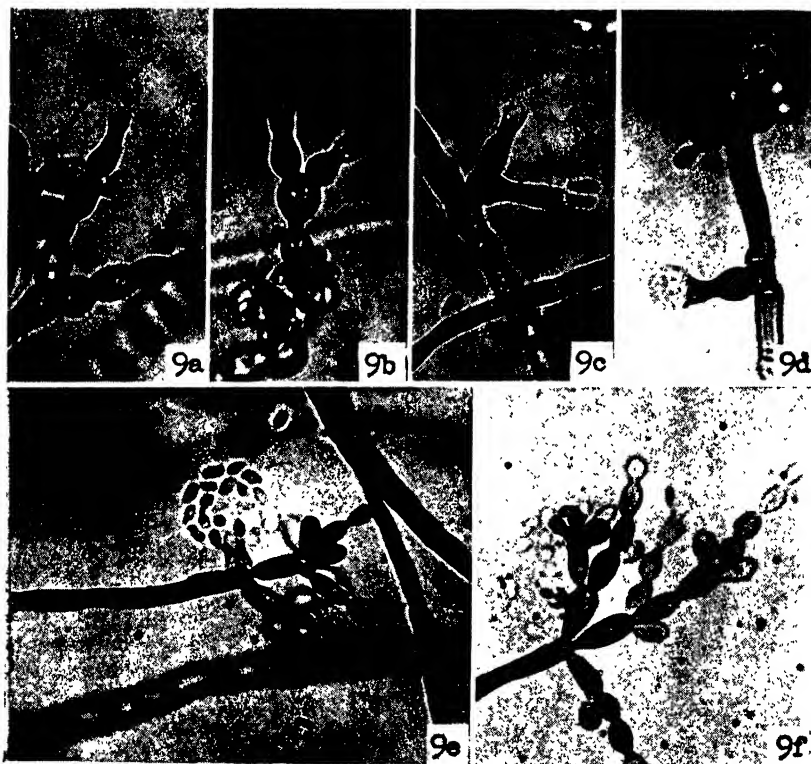


FIGURE 9. *Fonsecaea Pedrosii*: microscopic morphology representing the *Phialophora* type of sporulation. Note association of the *Phialophora* and *Hormodendrum* types of sporulation in d, e, and f.

ters showing tendency to chain formation as in *Hormodendrum* (FIGURES 8f and 8g). The morphology of the conidia resembles terminal and sub-terminal elements of *Hormodendrum* heads already described. The *Phialophora* type of sporulation (FIGURE 9) is usually scant to moderate in abundance; its morphology is similar to that of *Phialophora verrucosa* (q.v.).

Chlamydospores of the "sclerotic cell" type already noted in tissue, are sparsely produced in culture.

According to the various proportions in which the three methods of conidial formation just described may be represented in different isolates of

*Pedrosoi*, this species has been subdivided into four different varieties, namely: *Fonsecaea Pedrosoi* var. *typicus* Carrión, 1940<sup>18</sup>; *Fonsecaea Pedrosoi* var. *Cladosporioides* Carrión, 1940<sup>18</sup>; *Fonsecaea Pedrosoi* var. *Phialophora*, Carrión, 1942<sup>19</sup>; and *Fonsecaea Pedrosoi* var. *communis* Carrión, 1940.<sup>18</sup> The latter is the most common variety encountered, and its members always show an abundance of *Hormodendrum* and *Fonsecaea* sporulations. The *Phialophora* cups are sometimes as abundant as the other two types, but usually they are scant.

**Biologic Characters and Reactions.** It is generally admitted that *Fonsecaea Pedrosoi* requires an acid medium with a pH ranging between 5.5 and 6.5. The optimum temperature of this fungus has not been determined precisely, but its mycelium develops profusely, and sporulation is abundant at room temperature. A temperature of 100°C. is lethal in 15 minutes, while a temperature of 50°C. is well tolerated for at least one hour. The fungus grows best under aerobic conditions. There is no doubt, however, that it may grow to a certain extent at reduced oxygen tensions. Although *Fonsecaea Pedrosoi* may be artificially stained by different laboratory methods, it has shown no apparent reaction to Gram's method of staining. With the Ziehl-Neelsen technique, it takes on a darker and somewhat reddish color.

The biochemical activities of *Fonsecaea Pedrosoi* do not appear to be important. The fungus has been tested repeatedly for fermentative action against a large number of sugars with negative results. Other tests to determine its action on milk, gelatin, and serum are too few and conflicting to permit conclusions.

**Immunologic Reactions.** Meriin<sup>52,25</sup> claims to have induced specific, positive, intracutaneous tests in patients with chromoblastomycosis, using, as antigens, culture extracts of *Fonsecaea Pedrosoi*. *Fonsecaea Pedrosoi* is also capable of inducing the production of specific complement-fixing antibodies. This property has been determined in infected human beings by Balina *et al.*<sup>53</sup> and Martin *et al.*,<sup>31</sup> and in experimentally inoculated animals by Conant and Martin.<sup>54</sup>

**Response to Antibiotic and Chemical Agents.** (1) Alcohol. Experiments subjecting *Fonsecaea Pedrosoi* to the action of ethyl alcohol in different concentrations are being conducted in our laboratories. Although the observations are not yet completed, it may be stated that 95 per cent alcohol is lethal to all the isolates in 3 minutes, and to most of them in one or two minutes. (2) Sulfonamides. Keeney *et al.*<sup>30</sup> observed that sodium sulfamerazine produced a pronounced inhibiting action against *Fonsecaea Pedrosoi*. The use of this drug in a few patients with chromoblastomycosis, however, has not shown, so far, significant improvement. (3) Fatty acids. Keeney *et al.*<sup>55</sup> have also studied the effect of fatty acids on the same group of fungi *in vitro*, and have found that certain salts of these acids, particularly sodium caprate and sodium undecylenate, possess striking fungistatic and fungicidal action against *Fonsecaea Pedrosoi*. In view of the toxicity of these salts for the albino mouse, these authors do not recommend the internal administration of such salts in the treatment of the deep mycoses of man, until further animal experiments warrant their use. There is no

reason, however, why these new drugs should not be tried locally on the cutaneous lesions of chromoblastomycosis. (4) Penicillin. The sensitivity of *Fonsecaea Pedrosoi* to penicillin has been tested by Keeney *et al.*,<sup>56</sup> as well as by Fleming and Queen,<sup>57</sup> with negative results in both cases.

*Host Range.* Man is the only well-known natural host susceptible to infection with *Fonsecaea Pedrosoi*. It is possible that a spontaneous infection noted by Carini<sup>58</sup> in the lungs and kidneys of a batrachian (*Leptodactylus pentadactylus*) was produced by this or a closely related fungus. Rudolph<sup>4</sup> observed in Brazilian cattle another disease similar to human chromoblastomycosis, but no information is given regarding the true nature of the pathologic process.

Many investigators have endeavored to produce artificial infection with *Fonsecaea Pedrosoi*. Takahashi (1937)<sup>29</sup> reported on the reproduction of typical lesions of chromoblastomycosis in the healthy skin of a patient who was suffering from the disease and also in a volunteer not previously infected, by inoculating a suspension of *Fonsecaea Pedrosoi* (*Hormodendrum japonicum*) isolated from his patient. The induced lesions were consistent histopathologically with chromoblastomycosis and the etiologic fungus could be recovered in culture.

Attempts to produce artificial infection have been carried out on eight different species of experimental animals, namely, rats, mice, rabbits, dogs, monkeys, guinea pigs, pigeons, and frogs. Successful infections have been claimed in all but the last two species mentioned. From the experiments so far reported, it would seem that, among the different animals tested, the rat and the mouse are the species more susceptible to infection with *Fonsecaea Pedrosoi*, that infection may be effected by more than one route of inoculation and, finally, that the intraperitoneal, the subcutaneous, and the intratesticular are the most adequate routes to produce infection.

Infection in laboratory animals may be more or less limited in extent or it may be systemic, or even fatal, according to the method of inoculation. The lesions are nodular; variously sized, usually small; sometimes suppurating; single, few or numerous; cutaneous, or internal (liver, lungs, kidneys, peritoneum, testicles); when cutaneous, sometimes verrucous. The histopathology represents a granulomatous type of tissue reaction similar to that noted in human lesions, with the infecting parasite constituting a prominent part of the picture.

*Taxonomy.* Up to the present time, the species *Fonsecaea Pedrosoi* has shown no evidence of sexual reproduction and, consequently, it has its place among the Fungi Imperfecti. In view of the general morphology, sporulating habits, and dark color of the parasite, it has been included in the order Moniliales, family Dematiaceae. Finally, the simultaneous occurrence, in this organism, of three types of sporulation, which correspond to the genera *Hormodendrum*, *Phialophora*, and *Acrotheca*, respectively, has led to the classification of the species in the new genus *Fonsecaea* Negróni, 1936,<sup>20</sup> as emended by Carrión.<sup>18,19</sup> *Fonsecaea Pedrosoi* has been subdivided into the four varieties, *typicus*, *Cladosporioides*, *Phialophorica*, and *communis*.

The generic position of the "*Pedrosoi*" group has been a much debated subject. Differences of opinion have arisen mainly from (a) the triple

sporulating ability of the fungus, (b) the outstanding and misleading predominance of one of the sporulating types in each of the varieties *typicus*, *Cladosporioides*, and *Phialophorica*, and (c) failure to recognize the specific identity of these three varieties as established by the existence of intergrading forms (variety *communis*), which link them together and preclude any subdivision of the group beyond the ranks of varieties. The classification of *Pedrosoi* as a *Fonsecaea* has been accepted here on the following basis: (1) *Fonsecaea* Negroni, 1936 emend.,<sup>18,19</sup> is a legitimately created generic group including at least two well-differentiated species, namely, *Pedrosoi* and *compactum*; (2) it admits, *a priori*, the simultaneous occurrence of the

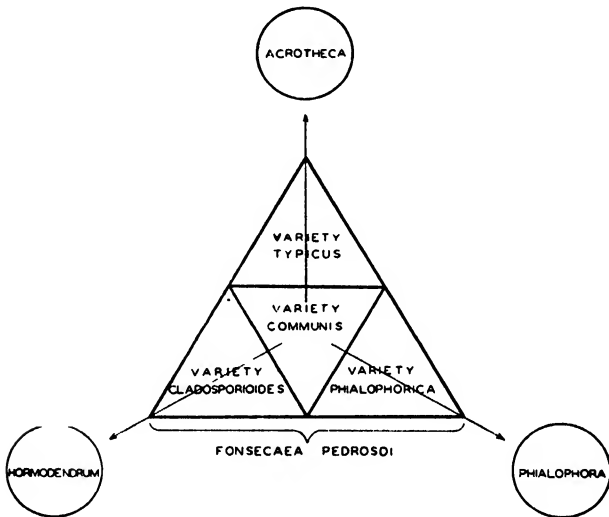


FIGURE 10. Diagram illustrating interrelations between the genus *Fonsecaea* and other related form genera. The large triangle represents a species of *Fonsecaea*, namely, *Pedrosoi*. The included smaller triangles are varieties of that species, and the circles represent the three genera to which *Pedrosoi* appears to be related, namely, *Hormodendrum* (*Cladosporium*), *Acrotheca*, and *Phialophora*. The arrows indicate suggested lines of evolution. *Fonsecaea Pedrosoi*, var. *communis* (center of triangle), possesses in conspicuous abundance the types of sporulation characteristic of the three genera above mentioned and, thus, would appear to represent their common origin. Different trends of evolution probably led to the development of the varieties *Cladosporioides*, *typicus*, and *Phialophorica*, each of which shows marked predominance of one of the types of sporulation, with equally marked reduction of the other two types. Further progress along the same line of evolution may have led to the differentiation of the three genera represented by circles, in which two of the sporulating types have disappeared completely, sporulation taking place, in each group, by a single method.

*Hormodendrum*, *Acrotheca*, and *Phialophora* methods of sporulation (FIGURE 10) as noted in its constituent species; (3) it covers without strain the different varieties of *Pedrosoi*; (4) it leaves out fungi that are exclusively *Hormodendrum*, *Acrotheca*, or *Phialophora* in type (FIGURE 10); (5) it recognizes the existence of a characteristic *Fonsecaea* method of sporulation which is present in all the known strains of the group (FIGURE 8), reaching its highest degree of development in *Fonsecaea Pedrosoi typicus* (FIGURES 8a and 8b); (6) as a generic name, it is neither misleading nor confusing; and, finally, (7) *Fonsecaea* has already become extensively popular, as noted in a review of recent publications on chromoblastomycosis.

In view of the fact that the species *Pedrosoi* is an imperfect fungus possess-



ing exceptional morphologic characters which link it closely to different form genera, it seems probable that differences of opinion regarding its generic position will continue to exist in the future. However, we believe that, with the broad concept of the genus *Fonsecaea* and the subdivision of *Fonsecaea Pedrosoi* into varieties, students in medical mycology will be able to work more effectively and lose less time in the classification of new or unknown isolates of this specific group. It should be understood that this taxonomic problem will not be definitely solved until the perfect phase of *Fonsecaea Pedrosoi* becomes known.

*Phialophora verrucosa*, Medlar, 1915.<sup>17</sup> *Synonymy.* *Cadophora americana* (Melin and Nannfeldt<sup>59</sup>).

*Geographic Distribution.* *Phialophora verrucosa* has been encountered in only six chromoblastomycotic infections. The majority of these infections occurred in continental United States, only two cases occurring abroad: one in Uruguay<sup>60</sup> (South America), and one in Algiers<sup>61</sup> (North Africa). Although the small number of *Phialophora* infections does not warrant final conclusions regarding the geographic distribution of the fungus as yet, it is of interest to note that five of the isolations were made in temperate climates, while only one, the North African strain, corresponds to a tropical climate.

*Natural Habitat.* Kress *et al.*,<sup>62</sup> who worked with material from the wood pulp industry in the United States and Canada, reported the isolation from this material of a dark-colored fungus which was first classed as *Cadophora americana*, but was later shown to be identical with *Phialophora verrucosa* by Conant and Martin.<sup>54</sup> Thus *Phialophora verrucosa* has become an additional member of the small group of pathogenic fungi so far discovered in their natural environment.

*Morphology in Pathologic Tissue.* In parasitized cutaneous tissue, the fungus is indistinguishable from *Fonsecaea Pedrosoi*.

*Gross Morphology in Culture.* Healthy colonies are produced in various laboratory media, with great variations in the rate of growth according to the individual isolate, cultures three weeks old in dextrose agar ranging in diameter between 0.5 and 3.5 cm. Gross cultural characters are generally more evident between the sixth and eighth weeks of growth (FIGURE 11a). The cultures are roughly conical, measuring 3.5 to 6 cm. in diameter on the eighth week, with a summit elevation of 5 to 10 mm. The surface is irregular, the border festooned or indented, and the aerial mycelium is profuse, short, gray, dark olivaceous or dark brown, forming a velvety or feltlike growth.

*Microscopic Morphology in Culture.* The microscopic characters are usually more conspicuous between the fourth and sixth weeks of growth. The vegetative hyphae are straight or cylindrical, branching, and septate, with articles 8 to 25 microns long by 2 to 6 microns in diameter, septation often closer, forming oidiod or moniliform mycelium; the cell walls are coarse and dark brown, with the cell membrane thin and hyaline; the protoplasm is yellowish-brown, finely granular, and contains refractile droplets; the nucleus is not apparent in fresh preparations but visible with hematoxylin stain. The conidiophores are either terminal or lateral (FIGURES 11b, 11c, and 11d)

and unicellular, measuring 5 to 12 by 2 to 3.8 microns, each conidiophore consisting of three parts: the base, largest of all, containing protoplasm and nucleus; a constricted portion, or neck; and a cup-shaped outlet, ranging in diameter, at the lip, from 1.1 to 4.7 microns, usually from 2 to 3. As a rule, the neck and cup form the terminal portion of the conidiophore, which thus becomes flask-shaped; otherwise, cup and neck arise laterally from fertile hyphal article. The conidia are semiendogenous, produced singly and successively by budding through the neck into the cup, often numerous, overflowing the cup and agglutinating to form spherical spore masses, loosely adherent to the cup (FIGURES 11b, 11c, and 11d). The conidial elements are oval, sometimes elongated. The surface is smooth, the wall thin and hyaline, measuring 0.8 to 2.8 by 1.4 to 7.8 microns, mostly 2 to 4 by 1 to 2. Chlamydo spores resembling sclerotic cells are noted in tissue, sparsely produced in artificial cultures.



FIGURE 11. *Phialophora verrucosa*: culture eight weeks old (a) developed at room temperature on Sabouraud's "milieu d'épreuve." Note lateral and terminal phialides, and tendency of conidia to agglutinate at the opening of the conidiophore (b, c, and d).

**Biologic Characters and Reactions.** *Phialophora verrucosa* thrives best on an acid substrate. The hydrogen ion concentration of the media used in cultural studies of this fungus ranges between 4.3 and 7. Its optimum temperature has not been precisely determined but the fungus is known to develop well at 37°C., and satisfactory cultures may be obtained at room temperature (24° to 33°C.). A temperature of 100°C. is lethal to the parasite in 15 minutes, while a temperature of 50°C. is well tolerated for at least one hour. The development of mycelium and sporulation in the depth of the substrate, especially in Czapek's agar, indicates that the organism may grow to a certain extent at reduced oxygen tensions.

Medlar<sup>12</sup> found that, in smears from cultures, the fungus elements would stain well, though diffusely, with all the routine bacteriologic stains, structural details being more evident after staining with dilute methylene blue for 10 minutes or longer, followed by thorough washing in water. It is

stated that, in fresh preparations of the mycelium stained with hematoxylin, practically all the cells revealed the presence of a nucleus, and, in the case of conidia, the nucleus also becomes visible with eosin and methylene blue. Young fungus cells would be Gram positive, while the older structures would take the blue irregularly.

According to Medlar,<sup>12</sup> *Phialophora verrucosa* would form no pellicle in litmus milk; the milk is not coagulated or peptonized and is gradually made alkaline. No indol is produced in Dunham's peptone solution, the medium becoming dark brown to chocolate brown in old cultures. The fungus is a nonfermenter of sugars.

Conant and Martin<sup>51</sup> demonstrated that the serum of a rabbit artificially immunized against *Phialophora verrucosa* possessed complement-fixing antibodies in high titer for this species and, in a lower titer, for the species *Fonsecaea Pedrosoi*. It was further shown that anti-*Pedrosoi* and anti-*compactum* rabbit sera, which produced intensely positive reactions with the respective homologous fungus antigens, would also react with *Phialophora verrucosa* in a comparatively lower titer.

*Taxonomy.* The species *Phialophora verrucosa* has not shown a sexual phase of reproduction in laboratory cultures and should be classed, therefore, among the Fungi Imperfecti. It is remarkable that the semiendogenous spores produced by this species resemble the spermatia noted in certain types of Ascomycetes. However, attempts to induce ascus formation by the pairing of different strains in laboratory cultures have not been successful (N. F. Conant, personal communication to the author).

The production of conidiophores from superficial hyphae at any point on the surface of the thallus places this fungus in the third order of Saccardo's classification, namely, the Hyphomycetales (Moniliales), while the dark color of the cultures sets it among the Dematiaceae. Since the conidia are dark and unicellular, the position of the fungus lies in the first division of this family, with the Phaeosporae. According to the method of conidial formation, it falls in the subdivision Chalareae. The species was placed in the new genus *Phialophora* Medlar,<sup>12</sup> at the suggestion of Thaxter, due to the agglutination of the conidia into sporangium-like masses at the mouths of the phialides. Finally, the specific name *verrucosa* was selected because the lesions produced by the fungus resemble those of tuberculosis *verrucosa* cutis.

*Fonsecaea compactum* (Carrión) Carrión, 1940, comb. nov.<sup>18</sup> *Synonymy.* *Hormodendrum compactum* (Carrión, 1935<sup>21, 22</sup>); *Phialoconidiophora compactum* (Moore and Almeida, 1936<sup>43</sup>); *Phialophora compactum* (Binford *et al.*, 1948<sup>48</sup>).

*Geographic Distribution.* Up to the present time there are only two recognized representatives of the species *Fonsecaea compactum*. One of them is the fungus originally discovered in a case of chromoblastomycosis in Puerto Rico,<sup>21, 22</sup> the other was isolated recently from the skin lesions of a patient in the state of Tennessee (U. S. A.).\*

\* This isolate was kindly sent to our laboratory by Dr. Norman F. Conant, of Duke University, North Carolina.

*Morphology in Pathologic Tissue.* The morphology of this fungus in pathologic tissue (FIGURES 12a and 12b) is essentially similar to that already described for *Fonsecaea Pedrosoi*.

*Gross Morphology in Culture.* Growth of the fungus is slow in all the usual laboratory media. On Sabouraud's *milieu d'épreuve* and 4 per cent glucose agar (FIGURES 12c and 12d), the fungus, when grown at room temperature, produces colonies that are roughly conical in shape, measuring about 2.5 cm. in diameter, with a summit elevation of approximately 6 mm. at the end of the sixth week. The center of the cultures forms an irregular mammillary prominence about which the colony slopes down unevenly toward a shallow marginal zone. The border is irregular and indented.

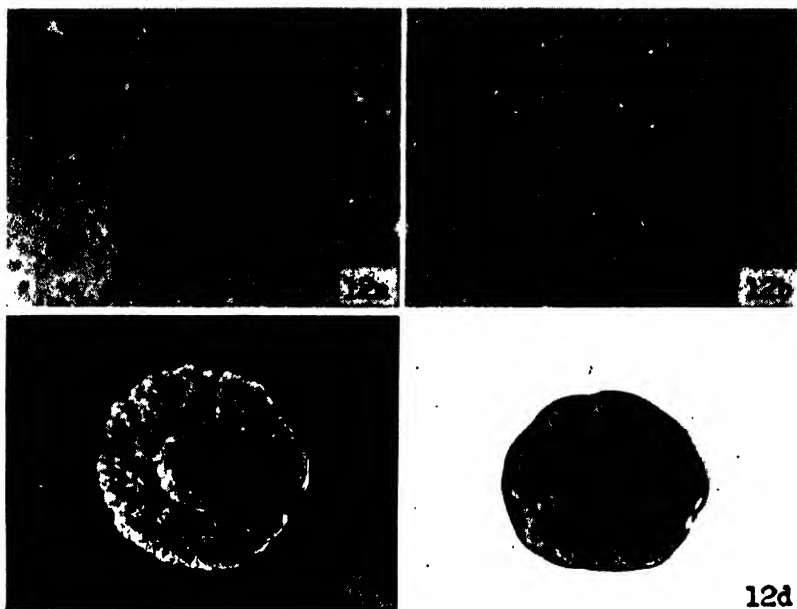


FIGURE 12. *Fonsecaea compactum*: (a) aggregate of fungus cells resembling sclerotium in the infected epidermal scales (note internal septation in some of the cells); (b) germination of fungus cells in the infected epidermal scales; (c) culture six weeks old developed at room temperature on Sabouraud's "*milieu d'épreuve*"; (d) culture six weeks old developed at room temperature on 4 per cent glucose agar.

The aerial hyphae are tufted, forming a plush-like or velvety brownish-black growth. On Czapek's agar the cultures are poorly developed, measuring 10 to 16 mm. at the end of the sixth week, with the mycelial growth chiefly in the substrate, the center of the colony showing a scant brownish-black, powdery, aerial growth.

*Microscopic Morphology in Culture.* Microscopic characters are usually more conspicuous after the third week of growth. Vegetative hyphae are long; coarse (2.5 to 5.2 microns); septate; branching, sometimes dichotomously; borders, usually irregular; cell walls, thick and dark; protoplasm olivaceous, granular, with refractile droplets; hyphal fusions, frequent; nucleus, not apparent in fresh preparations. The sporulation is of three dif-

ferent types; all occasionally combined in the same spore head. (a) The *Hormodendrum* type, which predominates (FIGURES 13a, 13b, and 13e). It has fertile branches, erect or ascending, often arborescent, and with terminal cell (conidiophore) swollen, often flask-shaped and darker, having, at the tip, broad facets to which spores are united. Its conidia are produced in short and profusely branching chains, some of the branches growing basipetally; the conidial elements are unicellular, spherical to subspherical, mostly barrel-shaped; the cell wall is smooth, coarse, and dark; the protoplasm is olivaceous and granular; and the disjunctors are absent, each

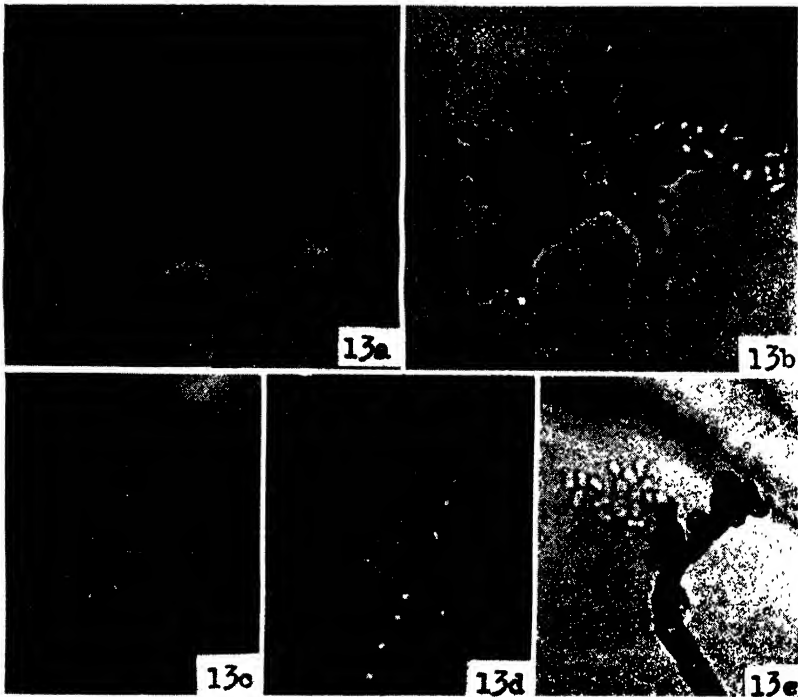


FIGURE 13. *Fonsecaea compactum*, microscopic morphology: (a) *Hormodendrum* type of sporulation; (b) conidial head of *Hormodendrum* type with one of its conidia transformed into a sporulating phialide; (c) *Fonsecaea* type of sporulation; (d) *Phialophora* type of sporulation; (e) combination of the *Phialophora* and *Hormodendrum* types of sporulation produced terminally on same hyphal structure.

conidium being compactly linked to its immediate neighbors in the chain by broad articulating facets. The conidia are 2.5 to 4.8 by 2.5 to 3.8 microns in size, basal elements 3.8 to 6 by 3 to 4.5 microns. (b) The *Fonsecaea* type, which is not conspicuously abundant (FIGURE 13c). Its conidiophores consist of an article, usually short, which is straight or irregular, more darkly pigmented than vegetative mycelium, disposed terminally, laterally, or intercalarily, and which is sometimes produced by transformation of the spore element in *Hormodendrum* spore head. The surface of conidiophore is irregular, due to the presence of broad facets to which spores are attached. Its conidia, individually, are similar in morphology to terminal and sub-

terminal elements of *Hormodendrum* chains already described in type (a) but produced acropleurogenously and singly (not catenate), forming clusters. Some clusters, however, show a tendency to chain formation, as in *Hormodendrum*. (c) The *Phialophora* type (FIGURES 13b, 13d, and 13e), which is scant, and has a morphology similar to that of *Phialophora verrucosa* (q.v.).

Chlamydospores resembling "sclerotic cells" as noted in tissue, sparsely produced in culture.

**Biologic Reactions.** *Fonsecaea compactum* requires an acid medium with a pH ranging between 5.5 and 6.5. Its optimum temperature has not been precisely determined, but it develops well at both room (25°C.) and incubator (37°C.) temperatures. A temperature of 100°C. is lethal to the parasite in 15 minutes, while a temperature of 50°C. is well tolerated for at least one hour. Although growth is usually more abundant under aerobic conditions, the development of mycelia in the depth of the agar, especially in Czapek's medium, indicates that this organism may grow at reduced oxygen tensions.

Conant and Martin have demonstrated that *Fonsecaea compactum* is capable of inducing the production of specific complement-fixing antibodies in rabbits by actively immunizing the animals against this fungus. The serum of the immunized animals showed a positive cross reaction against *Fonsecaea Pedrosoi*.

In a desiccated glucose agar culture of *Fonsecaea compactum*, which was kept in the laboratory under ordinary conditions, the fungus was found to be still viable after a period of over two years.

**Taxonomy.** The species *compactum* has failed to reveal a sexual phase of reproduction in laboratory cultures, and should be classed, at least for the present, among the Fungi Imperfecti. The production of conidiophores from superficial hyphae at any point on the surface of the thallus places it in the third order of Saccardo's classification, namely, the Hyphomycetales (Moniliales). Its familial position, as determined by the dark color of the mycelium and conidia, is among the Dematiaceae. It has been included in the genus *Fonsecaea* because it possesses the three types of sporulation characteristic of that genus, namely, the *Hormodendrum*, the *Fonsecaea*, and the *Phialophora*. The specific name *compactum* was given in consideration of the compact articulation of the spores in the *Hormodendrum* chains.

***Torula poikilospora.*** This fungus was isolated from purulent exudate and fragments of lesions in a case of chromoblastomycosis observed in Tokyo, Japan, and reported by Takahashi in 1937.<sup>63</sup>

According to the description of Takahashi, the characters of this parasite in pathologic tissue and its gross morphology in culture would be consistent with any of the species already described above.

The microscopic morphology of the fungus may be summarized as follows: vegetative hyphae straight, undulated, or bent at acute angles; cylindrical; profusely branched, some filaments showing multiple terminal branching; septate, with articles 3 to 25 microns long by 1.5 to 4.5 microns wide; protoplasm olive-green, older hyphae more deeply colored and containing variously shaped deposits of a black pigment. The fertile hyphae are undifferentiated. The arthrospores are developed at the tip and sides

of filaments, forming long, branching chains, the acrogenous chains being longer and more branched than the pleurogenous. The spores are firmly linked to immediate neighbors in the chain by broad articulations, and are olive-green, the older containing black pigment deposits. Some of the spores are spherical (2 to 9 microns in diameter) or oval (2.5 to 14 by 1.4 to 8 microns), but most are irregular in shape (2 to 2.5 by 1.5 to 16 microns), the irregularity resulting from development of knob-like or short filiform appendages on the tip or on the side, never on the base, of the spore cell. These appendages sometimes produce a secondary element and often are separated from each other and from the mother spore by septation.

Chlamydospores developed in old cultures, sometimes in chains; elements double-contoured, light brown to brownish-black; terminal or intercalary.

In our fungus collection, two isolates from cases of chromoblastomycosis show essentially the same morphologic characters described by Takahashi for *Torula poikilospora*. Careful examination of these isolates in culture, however, have revealed, though sparsely, the types of sporulation characteristic of *Fonsecaea Pedrosoi*. Consequently, our two isolates have been classed in the *Pedrosoi* group. We have been unable to obtain a culture of Takahashi's organism for comparative studies, but are inclined to believe that his isolate, like ours, is another variety of *Fonsecaea Pedrosoi*.

*Hormodendrum Species.* Chromoblastomycosis has been ascribed to authentic *Hormodendrum* species by Simson *et al.*<sup>32</sup> and O'Daly,<sup>33</sup> respectively, in South Africa and Venezuela. From the descriptions given by Simson and O'Daly, as well as from preliminary studies of these fungi carried on in our laboratories, it is quite evident that the two isolates are very similar and that they are capable of sporulating by only one method, namely, the *Hormodendrum*.

*Hormiscium dermatitidis.* This fungus was originally isolated from the lesions of a patient with chromoblastomycosis in Japan, and the fungus was described as a new pathogenic species by Kano in 1937.<sup>34</sup> This author has reported the reproduction of a typical nodule of chromoblastomycosis on the healthy skin of his patient by inoculating, locally, a suspension of *Hormiscium dermatitidis*. The histopathology of the nodule was similar to that of other lesions naturally developed on the patient's skin. Experimental infections were also produced on mice, guinea pigs, and rabbits, the inoculum consisting of a suspension of the fungus grown in artificial cultures.

The morphology of the parasite in pathologic tissue was essentially similar to that already described for other fungi causing chromoblastomycosis. Cultures six weeks old developed at room temperature on 4 per cent maltose agar, measured 8 mm. in diameter and were hemispherical in shape. They were coal black in color, with the surface finely granular and wrinkled and the substrate stained by black diffusible pigment. On 4 per cent grape sugar agar on mulberry, and on potato plugs, the growth was essentially similar to that on maltose agar. On peptone agar the growth was poor and, in glucose and maltose peptone water, loose floccular colonies developed at the bottom of the tube.

The microscopic characters were relatively simple and constant in many laboratory media employed for the study of the fungus. Cultures four days

old on "glucose-peptone agar" developed at 30°C., showed "short chains of spherical, oval, rod-shaped, clavate, or swollen cells"; branching was definite, though rudimentary, dichotomous, sometimes trichotomous, and would take place at the tip or side of the cell, often close to an intercellular septum. The cellular elements were of different sizes, the spherical forms measuring about 10 microns in diameter, the others ranging from 3 to 5 microns in width by 5 to 20 in length; the cell membrane was thick and dark, becoming double-contoured and more deeply pigmented with age; and some cells showed an internal septum perpendicular to the longitudinal axis.

Large chlamydospores of "sclerotic" type were present in old cultures. Some of these chlamydospores showed internal septation in one or more plains; some showed constriction at the line of contact of the cell wall with the internal septum; and some produced daughter cells by lateral sprouting.

The optimum temperature of the fungus appears to be between 20° and 30°C. At 37°C. the development was very poor, and at 43°C. it was inhibited.

*Candida-like Species.* This black, *Candida*-like fungus was repeatedly isolated by Berger *et al.*<sup>36</sup> from the cutaneous lesions of a Canadian patient who had been suffering from papillomatous growths over a period of fourteen years. Histopathologically, the lesions presented the classical picture of chromoblastomycosis, although the fungus was smaller, more delicate, and distinctly budding.

In the host tissues, the fungus appeared somewhat different in two series of biopsies done at different periods. In the earlier biopsies, the parasitic bodies were very abundant, rather small and delicate, and had fine membrane, some of them showing distinct budding, while others would form short, bead-like chains. In biopsies done at a later period, the fungus cells were not so abundant, but were coarser, thick-walled, irregular, devoid of budding, generally clustered, and often engulfed by giant cells.

In his description of the fungus, as originally isolated, Berger reports: "Colonies on corn meal and hay infusion agar, Loeffler's serum and blood agar, remain small (3 to 4 mm.), and die soon on the two latter media, but are larger on glycerolated potato and carrot, and on Sabouraud's agar (4 to 7 mm.), and still larger on Lowenstein's and Petragnani's media (up to 15 mm.). On Sabouraud's agar, they are dark brown from the beginning, and half-spherical; on the other media they are coal-black; they are always moist, of creamy consistence and glossy. . . ." "In potato water (Langeron and Talice), small, fluffy colonies rest on the bottom and the liquid contains a great number of single or budding yeast cells; there is no veil." "Cultures made from a later biopsy (1944) yielded brown to black colonies which grew much more slowly, were rough and dry. . . ."

Differences in microscopic morphology were likewise noted between the early and late isolations of the fungus. Berger states that cultures of the early isolations "at pH 7 are predominantly formed by yeast-like cells and of only a scant pseudomycelium; at lower pH's, pseudomycelial branching filaments extend on the surface and into the underlying medium, and bear clusters of pseudoconidial blastospores; there are no aerial hyphae." In cultures obtained from a later biopsy (1944), the "fundamental characters



are still yeast-like, but the cells are larger and thicker, often double-walled; outlines often irregular, and many elements show internal septations in different planes. The resulting multicellular structures become very complicated, and are made up of intricately intertwined, irregularly curved, and anastomosing, rather thick threads of clustered elements which grow in all directions." These "... cells may contain one or more round lipoid inclusions. . . ." They "may be considered" as "sclerotic cells and are obviously a variant of the initially yeast-like pseudomycelial fungus," and "In hanging drop and slide cultures, many budding cells may be seen which remain attached to the mother cell, and form, by successive buddings, moniliform strands of various lengths; the latter become pseudomycelial through elongation of the individual elements. Apical blastospores appear and give in turn rise to pseudomycelial threads, but the branches are few and the resulting arborization is, therefore, rather simple. Some blastospores show intensive budding of many small conidia-like blastospores, which form soon more or less large clusters of easily detached cells. There is no trace whatsoever of conidiophores or of any other specialized apparatus of sporulation, although many colonies were kept alive for over two years."

Berger's organism grows equally well at room and incubator temperatures; it does not grow in the depth of agar stabs. At pH 7, its morphology resembles that of the yeast, and at lower pH's, the cultures become filamentous.

The fungus does not liquefy gelatin and has no action on milk. It acidifies agar media containing glucose, fructose, mannose, galactose, cellobiose, and xylose respectively, but there is no gas formation in the corresponding liquid sugar media.

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## HISTOPLASMOSIS AND PULMONARY CALCIFICATION\*

By Amos Christie

*Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee.*

Over the years, the pediatrician and the radiologist have come to recognize that there are no pathognomonic changes of primary tuberculous infection in the chest roentgenogram. It can be stated unequivocally, however, that changes, when interpreted in view of the tuberculin test, size, extent, and location of the shadows, represent our best and most practical means for an accurate diagnosis of primary pulmonary childhood tuberculosis. Without these two diagnostic procedures, the pediatrician would be helpless in the differentiation of childhood tuberculosis from other conditions causing infiltration, exudation, and calcification. This is probably why he has been on the defensive in recent years, when it became apparent in everyday practice and particularly from mass Roentgen surveys that many children were shown to have massive and multiple calcification usually attributed to tuberculosis, though a considerable number of these were negative to large doses of old tuberculin.

The problem can be stated in another way. It has been considered generally by our colleagues in pathology that calcification in the lungs, the thoracic lymphatic channels, and the mediastinal gland structures is due almost invariably to tuberculosis. At the same time, most pediatricians and immunologists believe that tuberculin sensitivity is a relatively stable phenomenon. One of these views must be in error. This can be seen by the fact that in the past twenty years there have appeared in the literature at least twelve reports which show a consistent discrepancy between the tuberculin tests and the prevalence of pulmonary calcification. In these papers, summarized in our earlier reports,<sup>1</sup> 27 to 83 per cent of young individuals were found to have pulmonary calcification, a phenomenon generally accepted as indicative of tuberculosis. Yet, in each study, one-half or less of the individuals were found to have positive tuberculin tests. These reports, with few exceptions, were the outcome of studies of young people living in states bordering the Mississippi River and the states of the Western Appalachian slope.

While anergy or loss of sensitivity to tuberculin from complete healing of the tuberculous process may be an acceptable explanation in some cases, we do not believe that this can explain the marked prevalence of pulmonary calcification in this area. We were challenged to investigate other possible causes for these findings.

*Theory Behind Histoplasmosis as a Cause of Calcification.* Histoplasmosis in a previously unrecognized benign form was not too remote as a possible explanation for some of this calcification. This infectious disease caused by the fungus *Histoplasma capsulatum* occurs in all age groups and, from

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all reports, was uniformly fatal. This in itself struck us as being unusual. It can be of acute or chronic nature. No benign forms had been described. While it is of world-wide distribution, more than half of the reported cases have occurred in states which correspond to the area of benign calcification in nontuberculin reactors just discussed. It is due to a fungus which occurs in tissues in the yeast forms and ordinarily grows on culture media in the mycelial form. Demonstration of the fungus etiology of the disease was first obtained by the epochal cultural and transmission experiments of DeMonbreum<sup>2</sup> at Vanderbilt University Medical School. The organism produces lesions which simulate many common diseases. It produces ulceration of the mucosa and miliary lesions with caseous necrosis and granulomata which closely resemble tuberculosis. This variety of clinical manifestations makes it seem quite possible that benign forms may exist and that the clinical picture might be one such as described in coccidioidomycosis, with its primary complex going on to calcification much as in tuberculosis.

It had been previously shown by Cox and Smith<sup>3</sup> in 1939, and later by Aronson<sup>4</sup> in 1942, and it is generally recognized in the West that coccidioidomycosis produces a primary complex which goes on to produce calcification quite indistinguishable from that laid down by tuberculosis. Prior to these descriptions of benign form, *Coccidioides granuloma* was in somewhat the same state as histoplasmosis. It was almost uniformly fatal. Smith,<sup>5</sup> in 1943, postulated that the area of pulmonary calcification in nontuberculin reactors was also the endemic area of histoplasmosis. Infection with *H. capsulatum*, a fungus closely related biologically to *Coccidioides immitis*, might result in previously unrecognized benign forms of infection and the development of the calcifications described. These were the reasons behind our suspecting a benign form of histoplasmosis as a cause of our unexplained calcifications.

*Preliminary Clinical and Pathological Evidence to Support This Thesis.* In January 1944, a five-month old infant, suspected of having leukemia, was brought to Vanderbilt University Hospital. Subsequent studies proved that he had a miliary histoplasmosis. His blood cultures yielded as high as 3,000 fungus colonies per milliliter. This child and his parents reacted to histoplasmin: an extract of a culture of *Histoplasma capsulatum* which we had prepared.

Following this incident, we began to test all the children admitted to the pediatric ward with the same lot of histoplasmin and with coccidioidin. After approximately 125 children had been tested with both antigens, an analysis revealed that 23 per cent of the children reacted to histoplasmin, while only a few reacted, but equivocally, to coccidioidin. Though there were several very young children who had positive reactions, we were unable in any of them to obtain a history of an illness which might have caused the development of sensitivity.

Members of the pediatric house staff and medical student body who had lived all their lives in Kentucky, Tennessee, and Missouri reacted strongly to the antigen, while, conversely, other members who had recently arrived from California, Maryland, and New York did not react.

As a result of these preliminary skin tests with histoplasmin it seemed apparent: (1) that the response to the skin test was the result either of previous infection with *H. capsulatum* or of infection with some other fungus having a common or closely related antigen; (2) that infection with this unknown fungus was much more common than could be explained on the basis of any clinically known fungus infection, including the dermatomycoses; (3) that infection must at times be almost symptomless.

*The Infection: Histoplasmosis.* The literature has been filled with case reports of clinically recognizable histoplasmosis, starting with the cases of Darling<sup>6</sup> in 1906 and culminating with the reviews of Meleney<sup>7</sup> and of Parsons and Zарафонетис,<sup>8</sup> and new cases are being recognized continually. Certainly increasing knowledge of the disease has resulted in increasing diagnoses, to the point where it is no longer an extremely rare disease. While less than a hundred cases have been reported, it is safe to say that more are being recognized than are reported.

The clinical manifestations of the severe infections are protean. These may be epitomized by saying that there is irregular low-grade fever, hepatomegaly, and splenomegaly with anemia and leucopenia. There may be general glandular enlargement and frequently there are symptoms of pulmonary infection: pleural pain, cough, and expectoration. Many patients have had symptoms referable to ulcerative lesions of the gastrointestinal tract, including the mouth and oropharynx and genitalia. Destructive bone lesions occur. The duration of the disease in these patients is from a few weeks to as long as 15 to 20 years, and, in the great majority, the infection has been considered ultimately fatal. The diagnosis in these cases can usually be established by adequate cultural techniques and/or the examination of biopsy material, lymph nodes, and sternal marrow.

It is perhaps significant that 18 cases of proven histoplasmosis have been recognized at Vanderbilt Hospital (as of January, 1948) and that tissues from a half dozen other cases, culled from a group of nonspecific granulomatous lesions and from tuberculin negative cases with morphological diagnoses of hilar tuberculosis, are highly suspicious. While these cases are of great academic interest, they are, from our viewpoint, much less important than those patients who have minimal, nonfatal, almost nonsymptomatic infections.

Since the relationship of pulmonary calcifications to histoplasmin sensitivity was first pointed out, we, and others, have been engaged in a search for minimal infections. In this problem one is faced with the same difficulty that would be encountered, after 65 years of extensive investigations, in attempting to prove bacteriologically the existence of minimal primary tuberculosis. In fact, we have found that an additional number of technical difficulties arise which make the solution of the problem a tedious labor.

There follows a series of cases which we believe represent the benign or minimal form of the disease.

*Case 1.* A well-developed and nourished ten-month old infant with bilateral subdural hematomata died from the hyperpyrexia following an attempt at excision of the membrane. In the lower lobe of each lung

there was a small 3-4 mm. calcified nodule and the hilar lymph nodes were slightly enlarged, though free from caseation. Microscopic sections of the nodes and pulmonary granulomatous lesions failed to show tubercle bacilli but did show tubercle-like focal granulomatous lesions from which *H. capsulatum* was cultured.

*Case 2.* A three-month old female infant was admitted to the Vanderbilt Hospital with convulsions, bulging fontanelle, and signs of meningeal irritation. The family history was negative for tuberculosis. A spinal puncture revealed pneumococci and, in spite of adequate support-

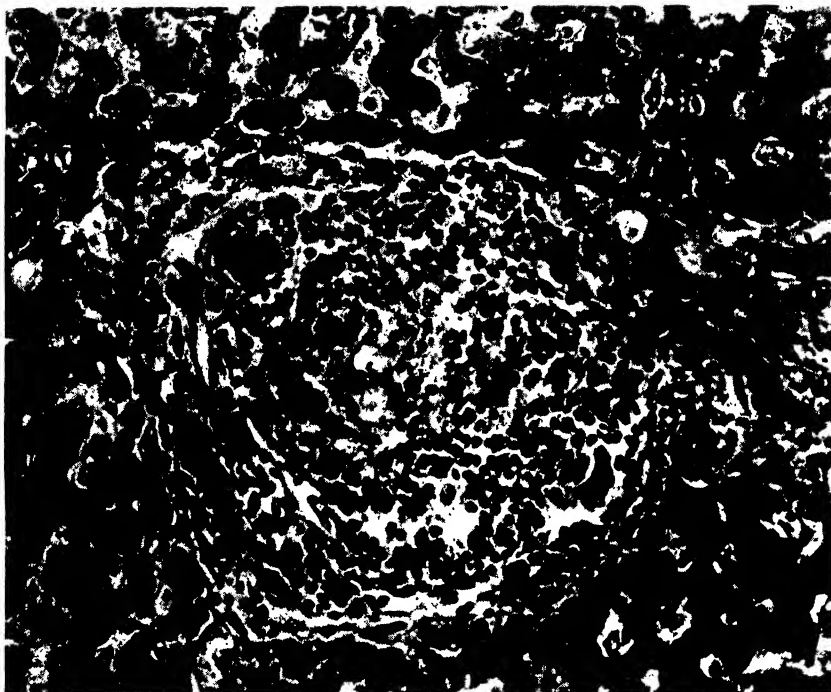


FIGURE 1. Liver ( $\times 300$ ). Granulomatous Lesion (Case 1).

ive and specific treatment with chemotherapeutic and antibiotic agents, she died.

The tissues and guinea pig inoculation were negative for acid-fast organisms. The lungs and spleen showed many chronic granulomatous lesions with evidence of healing. *H. capsulatum* was cultured from the pulmonary lesions.

*Case 3.* A male infant, age eight months, was admitted to Vanderbilt Hospital for hydrocephalus associated with a meningocele. The family history was negative for tuberculosis. During a spinal puncture for purposes of decompression of the increased intracranial pressure the infant suddenly expired.

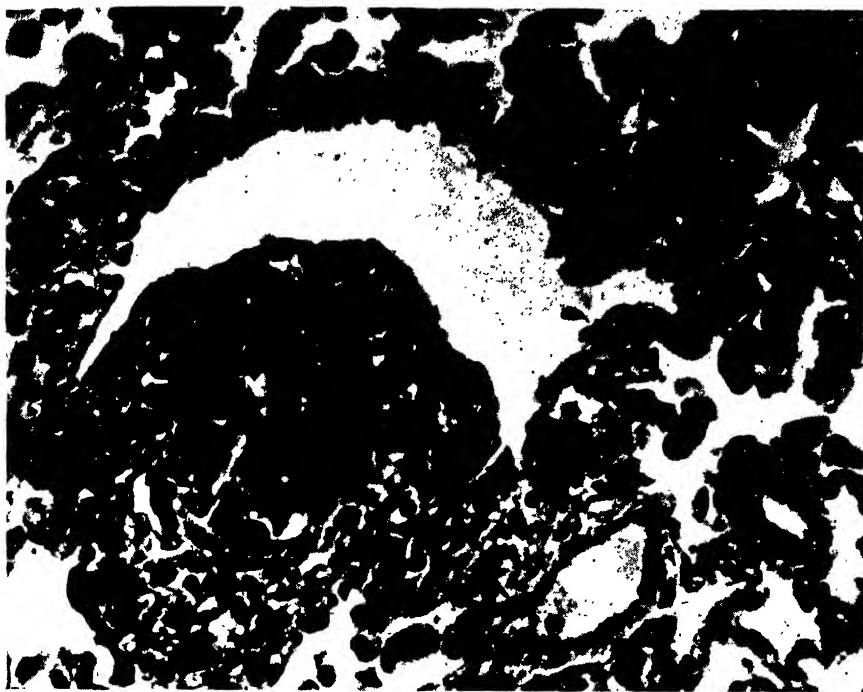


FIGURE 2. Lung ( $\times 375$ ). Showing a tubercle-like focal granulomatous lesion (Case 1).

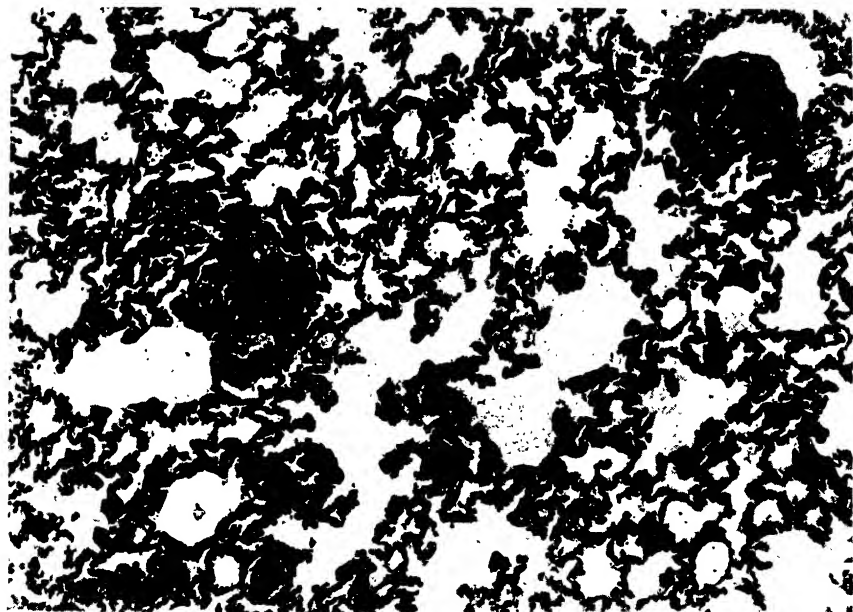


FIGURE 3. Lung ( $\times 120$ ). Showing tubercle-like focal granulomatous lesions (Case 1).



These sections were negative for acid-fast organisms. They did show a focal or interstitial pneumonitis, and the yeast forms of *H. capsulatum*

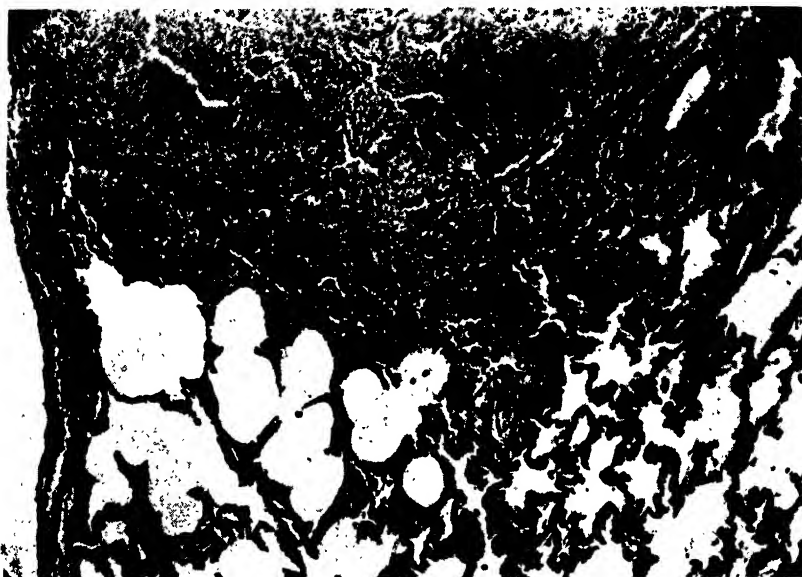


FIGURE 4. Tubercle-like lesion in lung ( $\times 120$ ), from which *H. Capsulatum* was cultured (Case 2).



FIGURE 5. Tubercle-like focal granulomatous lesion in lung ( $\times 120$ ), from which *H. Capsulatum* was cultured (Case 2).

in large mononuclear cells could be demonstrated. These were easily found in the edge of the tubercle-like lesions seen throughout the lung.

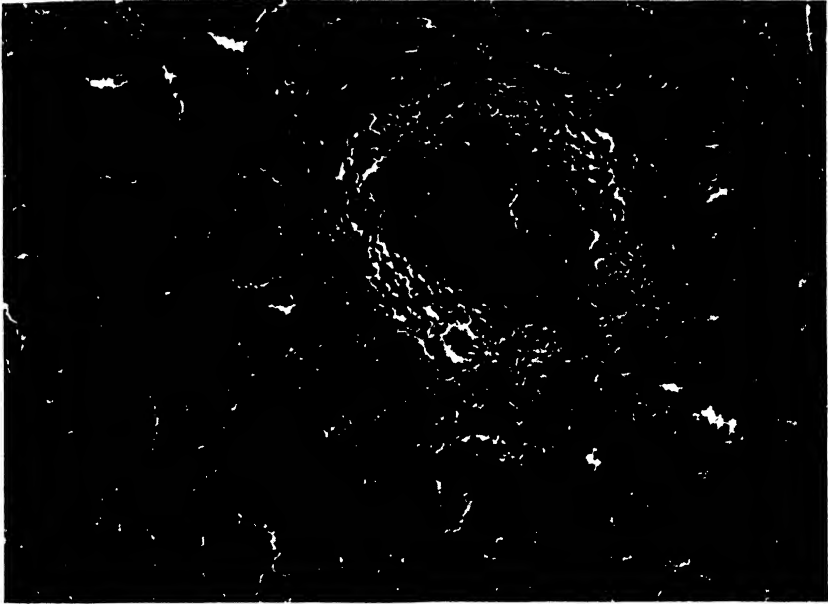


FIGURE 6. Tubercle in spleen ( $\times 120$ ) (Case 2).

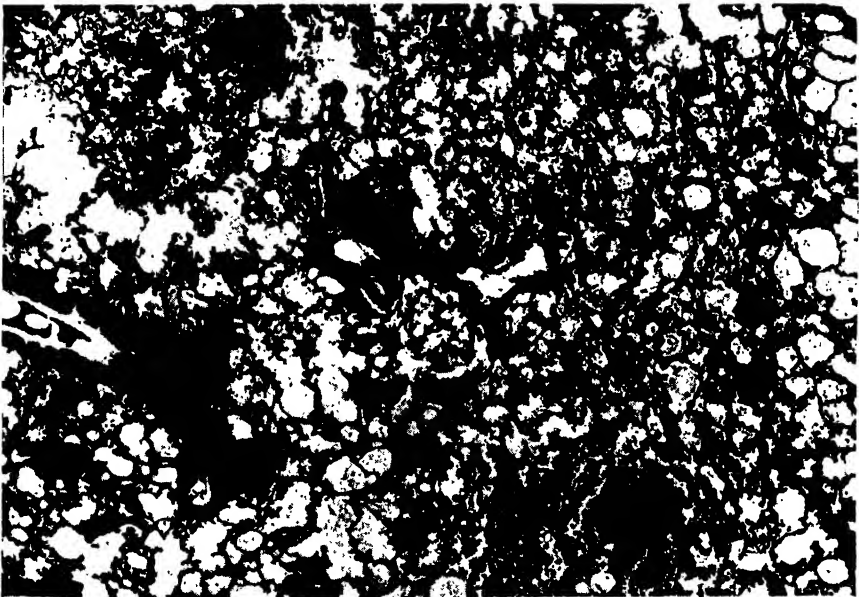


FIGURE 7. Lung ( $\times 120$ ) showing focal pneumonitis (Case 3).

We have recently cultured *H. capsulatum* from a hyperplastic mediastinal node with some central caseation recovered at autopsy from an infant who

died a postoperative death from congenital biliary atresia. We have also found *H. capsulatum* in biopsy material of two cases of chronic cervical adenitis. These cases will be reported in detail in a subsequent publication now in preparation.

While this work is still in a preliminary phase, it gives excellent promise of establishing satisfactory biological proof of the existence of minimal infections. Surely the repetition of these findings has reached a proportion where they could not all be early disseminating cases. These, then, we believe, are cases of minimal or benign histoplasmosis proven both histologically and culturally.

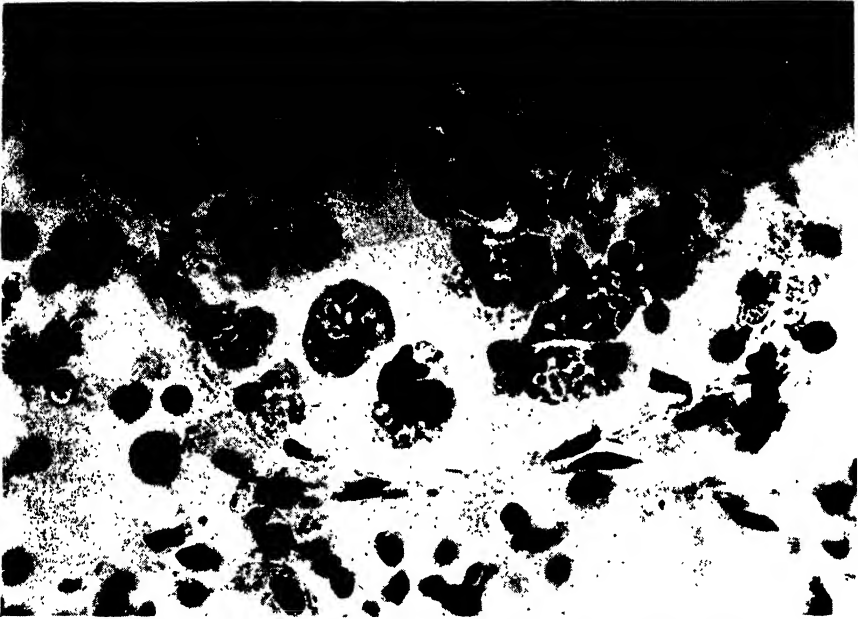


FIGURE 8. Yeast cells in mononuclear phagocytes of lymph node ( $\times 500$ ) (Case 3).

We have been trying to culture the organism from sputum or gastric washings. In tissues, *H. capsulatum* yeast cells are found almost entirely intracellularly and may multiply chiefly if not exclusively within the cells. Thus, unless the lesion goes on to caseation and ulceration, one cannot expect the release of organisms; and the sputum or gastric washings would be negative. This expectation has been confirmed in several ways. We have been unable to demonstrate or cultivate the organisms from the sputum in a single case with a fatal miliary infection and have failed, in other instances of what seemed to be almost certain infections, to show organisms in the sputum and/or gastric washings.

Early attempts to cultivate the organisms from autopsy materials *in vitro* and by passage through experimental animals were unsuccessful—the first because of the invariable contaminants; the second because the or-

ganism either was often of such low virulence or was no longer viable to the extent of producing infection, because of the action of the tissues. Recently, my associates<sup>9, 10</sup> at Vanderbilt University School of Medicine succeeded in cultivating the organisms from the lung and lymph node lesions of minimal cases, such as I have shown here.

It is our thesis, therefore, that infection with *H. capsulatum*, like *Coccidioides immitis* or the tubercle bacillus, can result in overwhelming infection with dissemination and fatal outcome. Depending on the resistance of the host, the virulence of the organisms, or other unknown factors, the organisms are rendered nonviable or phagocytized, as seen in one of the cases shown. Giant cell formation around the liberated lipid substances is followed by caseous necrosis with healing and calcification. We have seen calcification in a four-month-old infant who died of disseminated histoplasmosis. During these later states, it will be unlikely that the organisms are viable, and we should not expect to be able to get positive cultures. We feel that the focal, interstitial, or atypical pneumonitis cases of early life will be more fruitful for finding the organisms culturally or in tissues. This correlates with the age distribution for the development of sensitivity to the skin test material which we have shown before.<sup>1, 11, 12</sup>

*Histoplasmin Sensitivity and Pulmonary Calcification.* In the five years since the relationship of histoplasmin sensitivity to pulmonary calcification was first shown, the aroused interest has resulted in the publication of a considerable number of papers on the subject. Also, considerable effort has been expended in attempts to demonstrate a benign type of infection with *Histoplasma capsulatum*. We will now attempt to review what we think is significant of this material and to discuss the specificity of the antigen.

*General Considerations.* Preliminary studies with histoplasmin tests by members of the Department of Pediatrics at Vanderbilt University in 1945 showed that histoplasmin sensitivity was not uncommon and that such sensitivity was sometimes associated with pulmonary calcifications not attributable to tuberculosis. Palmer,<sup>13</sup> studying nurse cadets, was able to show a high degree of correlation between pulmonary calcification and histoplasmin sensitivity. He showed that, while only 25 per cent of the group as a whole reacted to histoplasmin, 85 per cent of those with pulmonary calcification reacted to this antigen. Our studies<sup>1, 11, 12</sup> have likewise shown that 87 per cent of Tennessee residents with calcification react to histoplasmin, though such sensitivity is only half that common in those not having calcifications. Only 18.8 per cent of those with calcifications react to tuberculin, while 18.6 per cent of the group as a whole react to this antigen.

On the other hand, Olsen, Bell, and Emmons,<sup>14</sup> in a study of the residents of Loudoun County, Virginia, were unable to find a positive association between a reaction to histoplasmin or tuberculin and the presence of pulmonary calcification, though there was a correlation between residence in a tuberculous household and the presence of pulmonary calcification.

Waring and Gregg,<sup>15</sup> using our histoplasmin, made a study of children in Charleston, South Carolina. They found only eight children with pul-

monary calcification and a negative reaction to tuberculin in a group of 349 children. Of the eight, five, or 62 per cent, reacted to histoplasmin, though they found positive histoplasmin reactions in that area in only two of 121 random tests, or 1.6 per cent. It is of further interest that the five histoplasmin reactors, among the eight with pulmonary calcification and negative tuberculin tests, either were natives of areas of high incidence of histoplasmin sensitivity or had made extended visits to such areas.

McWeeney, Crowe, and Dunleavy,<sup>16</sup> in a study of 320 Irish children, found no calcifications in 191 tuberculin negative children, nor did they find a single reactor to the histoplasmin which we supplied in the 320 children studied.

Alman and Opinsky<sup>17</sup> found 16 reactors to histoplasmin in 441 children from the District of Columbia area. Of these reactors, thirteen, or 81 per cent, had pulmonary calcifications. They do not, unfortunately, give the prevalence of such lesions in the group as a whole.

These studies, in general, show that pulmonary calcifications, in certain localities are statistically related to histoplasmin sensitivity, and that pulmonary calcifications are much more prevalent in communities where histoplasmin sensitivity is highly prevalent.

*Specificity of the Antigen.* Any conclusion based on skin testing inevitably brings up the question of specificity of the antigen. The recent studies of Emmons, Olsen, and Ethridge<sup>18</sup> have cast considerable doubt upon the specificity of the histoplasmin test. From these observations in animals and in older age groups, one might say that histoplasmin gave frequent nonspecific reactions; that it may well serve as an index of sensitization to various other fungi. We have from our earliest studies thought that such a situation might exist; that the histoplasmin sensitivity might be indicative of more than *H. capsulatum* infection, and that it might be indicative of sensitization by *H. capsulatum* and also by other agents having an antigenic complex closely related to that of *H. capsulatum*.

The studies of Emmons just mentioned showed that there was a marked degree of cross-reactions with histoplasmin in heterologous fungus infections. He has, accordingly (and quite logically), cast doubt on the question of histoplasmosis being responsible for the pulmonary calcifications observed. Also, he and his associates were unable in the survey of Loudoun County, Virginia, to find any evidence for the existence of a mild form of histoplasmosis. Howell,<sup>19</sup> however, in a recent study, has shown that different lots of histoplasmin vary markedly in their critical titers, a phenomenon which we have observed. He also showed that good antigens used at their critical titers yielded very few cross-reactions with heterologous fungus infections. These studies serve to emphasize the need for improving and standardizing histoplasmin preparations.

Yet our studies yield information which makes it seem quite certain that the reactions observed are truly specific allergic phenomena rather than reactions to nonspecific irritants contained in the preparation. The following points seem of importance in respect to this. (1) The broth, while containing foreign proteins, does not cause reactions with appreciable frequency to amounts of 0.001 cc. (2) Animals infected with *H. capsulatum*

give positive reactions but controls do not react. (3) Pilot studies<sup>11</sup> in 281 persons with coccidioidin and in 437 persons with haplosporangin, while yielding many positive reactors to histoplasmin, failed to yield a single definite reactor either to coccidioidin or to haplosporangin. It would seem that, while there might be cross-sensitivity in experimental animals, these two fungi are not responsible for much of our problem in the area described above. We are accumulating evidence that the same can be said for blastomycin. (4) The question of *Oidium albicans* has been suggested by Doctor Emmons and others as a possible explanation for some of our sensitivity to histoplasmin. The failure to find many young infants sensitive to histoplasmin would make it seem very unlikely that infection with the thrush organisms can be of real significance in relation to histoplasmin sensitivity. Oral thrush is a common infection of infants in this section and would, if it produced cross-sensitivity reactions, be likely to yield a high percentage of infantile reactors. Furthermore, thrush is not a commonly recognized infection in the older age group where we commonly find histoplasmin sensitivity. (5) Age distribution. The pattern of age response is such as one would expect from a widely disseminated infectious agent.<sup>12</sup> Infants are generally insensitive. The only positive reaction we have encountered in a child less than six months of age was in an infant dying with proven histoplasmosis, and we have seen only one other positive reaction in an infant less than a year of age. The rapid increase in sensitization in the next few years is certainly not correlated with any particularly developmental achievement. It is rather like the development of Schick negativeness in the nonimmunized urban groups, a phenomenon no longer generally believed to be due to natural development but generally attributed to immunization resulting from subclinical infections. (6) The geographic distribution of histoplasmin sensitivity offers strong support for the supposition that the reaction is a response to an infectious process. Geographical variations in the prevalence of pulmonary calcifications have been shown by a number of investigators, most strikingly by Long and Stearns.<sup>20</sup> They showed that Army inductees from the Western Appalachian slope and the bordering states west of the Mississippi and north of the Ohio River showed a greater prevalence of pulmonary calcifications than those from other areas. The studies of Palmer<sup>13</sup> and our studies<sup>1, 12</sup> have shown that histoplasmin sensitivity is much more prevalent in this area of excessive pulmonary calcifications. The contrasting study of Waring and Gregg<sup>15</sup> seems of great significance in this respect. They found pulmonary calcification in only 3.4 per cent of their school children in an area where the index of histoplasmin sensitivity was low, as contrasted with over 40 per cent in the group reported by Olsen, Bell, and Emmons,<sup>14</sup> 55 per cent in our study groups, and 12.7 per cent in the group studied by Furculow, High, and Allen,<sup>21</sup> the latter groups being from areas of high prevalence of histoplasmin sensitivity.

We believe that these studies clearly indicate an intimate relationship between pulmonary calcification and histoplasmin sensitivity in the areas where such sensitivity is highly prevalent.

Histoplasmin sensitivity prevalence has been shown in the foregoing to

vary greatly with geographical areas. We can also add that, in addition to the absence of reactors in Ireland,<sup>16</sup> none of several hundred individuals living in Holland reacted to histoplasmin; there were no clear-cut positive reactions in more than a hundred persons tested in Curacao; and positive tests are encountered very infrequently in children in New York City, Buffalo and Rochester, New York, in Detroit, in New Orleans, and in San Francisco<sup>22</sup>.

It has also been observed in Tennessee that sensitivity to histoplasmin is more prevalent in areas adjacent to streams and other areas of dampness than it is on high dry ridges.<sup>23</sup>

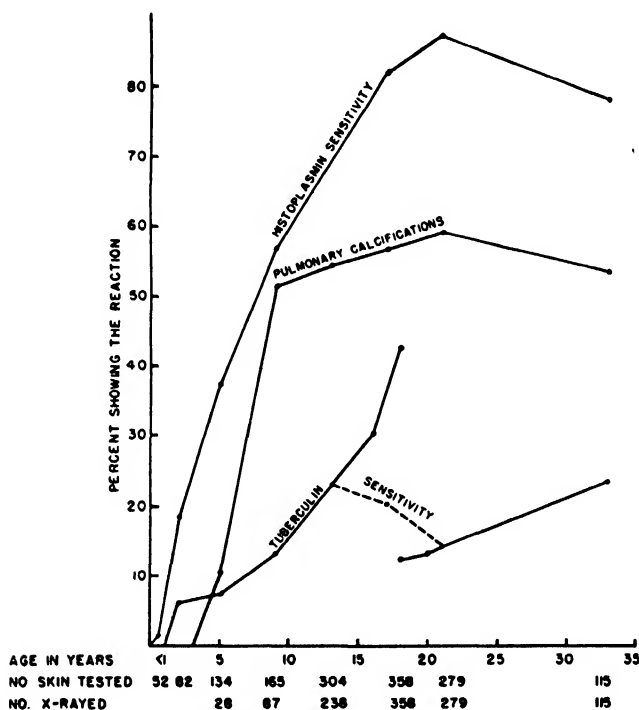


FIGURE 9. Age relationship in the development of histoplasmin sensitivity, pulmonary calcification, and tuberculin sensitivity.

From these studies it is apparent that the question of histoplasmin sensitivity and its relationship to pulmonary calcifications is one which may be of great importance in one locality and of no importance in another; and also that some factors of climate and physical geography must be of great significance in the epidemiology of histoplasmin sensitivity.

Our studies<sup>12</sup> show that there is a wide variation in the prevalence of sensitivity from one area to another within the state of Tennessee and in neighboring states. Indeed, the extensive studies reported by Palmer<sup>24</sup> and by Prior and Allen<sup>25</sup> showed that there was a similar variability in other states.

Furthermore, the wide variation in sensitivity observed in our studies in students from different states shows that the variation is in relation to a fairly definite geographic focus and is suggestive of an infectious agent existing commonly in an area where climatic factors favor its propagation.

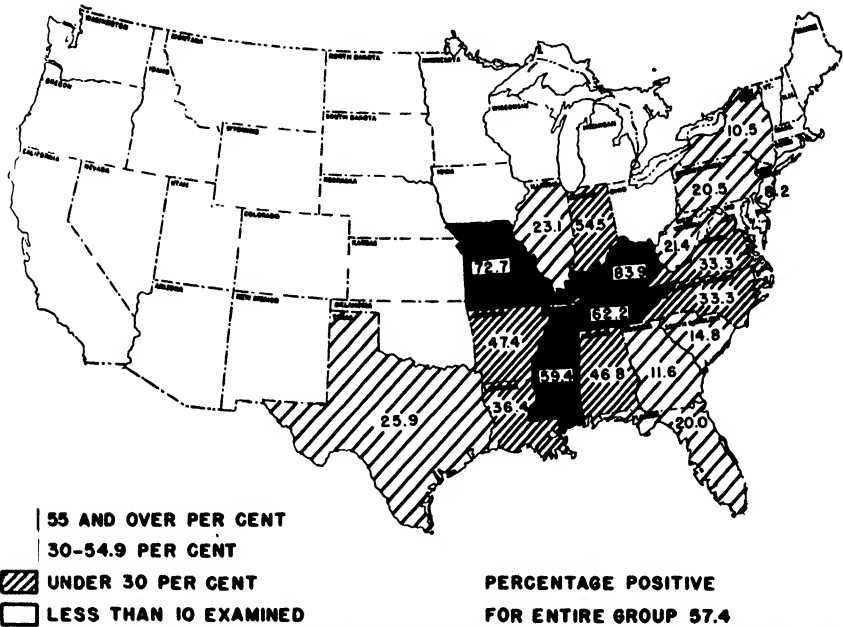


FIGURE 10. Distribution by state of residence of percentage of persons, examined at Vanderbilt University in 1945, reacting to histoplasmin.

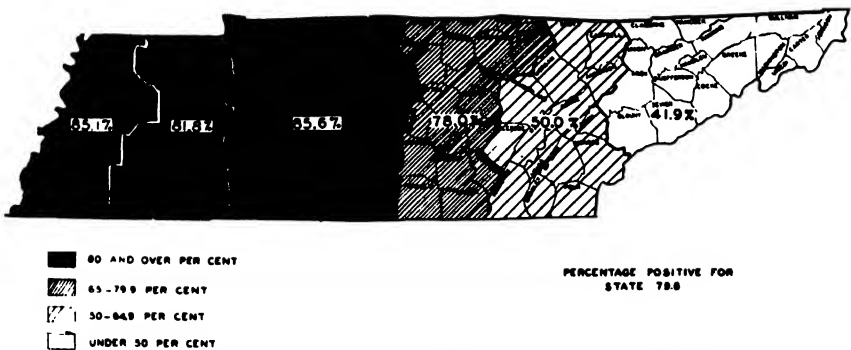


FIGURE 11. Percentage of persons examined at Vanderbilt University and Tennessee Industrial School reacting to histoplasmin, by county of residence, 1945.

Indeed, throughout these studies, wherever histoplasmin sensitivity is high, pulmonary calcification is also high. A similar relationship has been recognized in the problem of coccidioidomycosis, an infection in which sharply delimited foci of propagation and infection have been demonstrated.



None of these studies prove a direct causal relationship between pulmonary calcifications, histoplasmin sensitivity, and the disease, histoplasmosis. The histoplasmin sensitivity may be merely an index of the over-all problem of pulmonary and systemic mycotic infections. It may

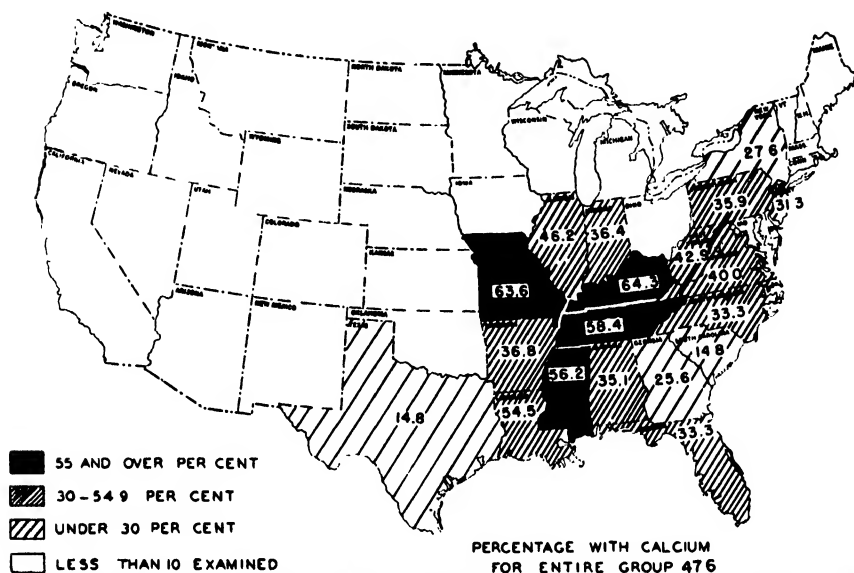


FIGURE 12. Distribution by state of residence of percentage of persons, examined at Vanderbilt University in 1945, found to have calcification.

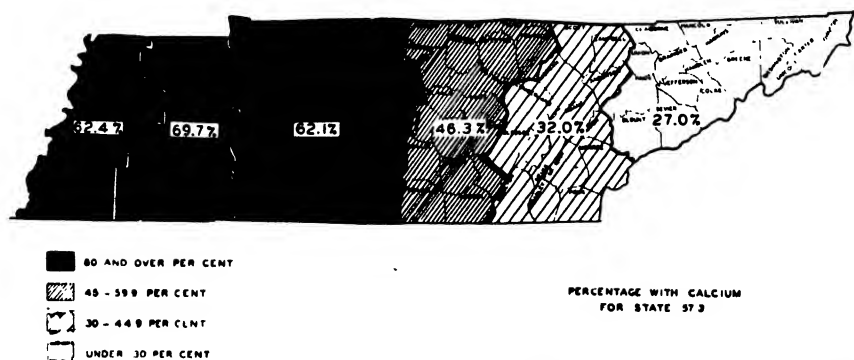


FIGURE 13. Percentage of persons examined at Vanderbilt University and Tennessee Industrial School found to have calcification by county of residence, 1945.

explain only a segment of our problem of pulmonary calcification in non-reactors to tuberculin. Further studies of cross-sensitivity among fungus antigens and infections are needed. Further study to establish the exact etiologic agent or agents responsible for the histoplasmin sensitivity and pulmonary calcifications is imperative. My associates, Doctor Peterson

and Doctor McVickar, and I are engaged in these studies at the present time.\*

Another approach to the solution of the problem of the minimal infection has been the study of pulmonary roentgenograms in children who have recently developed histoplasmin sensitivity. Infants being followed in well-baby clinics are given repeated histoplasmin tests and as sensitivity develops roentgenograms are made. In a number of these infants, we have been able to show pulmonary lesions which were not giving rise to significant symptoms. These infants have not been followed long enough to determine whether these lesions go on to calcification. In two instances, we have observed hilar lymphadenopathy of a degree sufficient to produce atelectasis and cough in young children who were repeatedly tuberculin negative and who had strongly positive histoplasmin tests.

We believe that these cases represent minimal infections with a fungus capable of producing histoplasmin sensitivity and which is presumably histoplasmosis in a benign form.

*Discussion and Summary.* These findings seem to have definite significance from the viewpoint of the interpretations of the mass roentgenographic studies now being made and contemplated. It seems quite certain that in states bordering the western bank of the Mississippi River and the states of the Western Appalachian slope a large part of the pulmonary calcifications are due to some infection other than tuberculosis. It seems that, while this relationship is most striking in the area outlined above, it is not confined to this area but has a much wider application.

If these studies have no other value, they do serve to reinstitute and re-emphasize the necessity of employing either tuberculin tests or the demonstration of tubercle bacilli in establishing the diagnosis of tuberculosis in minimal and noncavitated pulmonary lesions and in the healing lesions with pulmonary calcifications. Present-day concepts of the primary complex, or first infection tuberculosis, in its relationships to pulmonary calcification, need re-evaluation. The same may be true of reinfection tuberculosis.

*Conclusions.* (1) The problem of pulmonary calcification in tuberculin negative individuals has been reviewed. (2) The available epidemiological evidence and experience with histoplasmin skin tests to support this thesis have been reviewed and evaluated. (3) The significant clinical and pathological evidence suggesting that benign forms of histoplasmosis may be responsible for a segment of this previously unexplained calcification is presented. The significance of these observations has been stated.

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## CRYPTOCOCCOSIS AND BLASTOMYCOSIS

By Rhoda W. Benham

Department of Dermatology, College of Physicians and Surgeons, Columbia University,  
New York, N. Y.

The two diseases which form the title of this report were described for the first time in 1894: one from Europe, the other from the United States. These two, with a number of other distinct diseases, did in the past and in some cases still do appear in the literature under the name "blastomycosis." The resulting chaos in terminology has been difficult to overcome. It originated with the practice of referring to organisms reproducing by buds or blastospores as "Blastomycetes," and the conditions in which they occurred as "blastomycosis." Since the etiological agent in many different deep-seated fungus infections appears in the tissues or exudate from the lesions in the form of oval or rounded budding cells, they were often erroneously described as blastomycosis. Cultures proved, however, that a number of different fungi were involved and that a number of distinct diseases had been described under one name. Some workers went so far as to suggest that blastomycosis was a disease entity due to a plurality of species. Castellani,<sup>1</sup> for one, stated in 1920 that "Blastomycosis is a term generally applied to affections due to fungi of the genera *Saccharomyces*, *Cryptococcus*, *Monilia*, *Oidium*, and *Coccidioides*."

As a result, then, of cultural studies and of a careful consideration of the characteristic appearance of the fungus in the parasitic state, we now have a number of deep-seated fungus infections described under their respective names, and the term blastomycosis has become more limited in scope. The general practice is to restrict its use to the disease first described as such, namely, that of Gilchrist. Although Castellani<sup>2</sup> and Conant<sup>3</sup> have pointed out that the name is not suitable, neither advises dropping it. It has come to stand for a definite entity in medical mycological literature and has not much significance otherwise.

History shows us that the name *Blastomyces* was first given by Constantin and Rolland<sup>4</sup> (1888) to a fungus which they isolated from dung and described as a new species. It is a filamentous, branched form with conidia at the ends of short branches. These conidia or buds, as these authors called them, in turn produce so-called secondary buds at many points, and these, still others, until each fruiting branch transforms itself into a powdery spore mass. This fruiting mass is yellow to sulphur color, passing to red-orange and sometimes green. Only one species is known, *Blastomyces leucaeus*. This is listed as being in the collection at Baarn, but I have not as yet obtained a subculture. Just what it is would be anyone's guess, but, in the illustrations, the conidia are not unlike those of some of the gymnoascus forms, or of some of the dermatophytes. Certainly it is not a yeast-like fungus. In Clements and Shear's<sup>5</sup> key to Saccardo's classification we find it defined as follows: "Hyphae elongated and distinct from conidia. Conidia bearing hyphae of two sorts, the upright alone denticulate."

Just where or how the term *Blastomyces* entered the literature to designate those forms reproducing by a process of budding or by blastospores is not quite clear, but Gilchrist and Stokes<sup>6</sup> appear to have been the first to use it in the medical literature, when they gave the name "*Blastomyces dermatitidis*" to the fungus and the name "Blastomycetic dermatitis" to the cutaneous disease which they described. The fact that rounded, budding cells were noted in both their case and the European case described by Busse and Buschke led then to their both being called "blastomycosis." We now know, and it has been pointed out many times, that the organisms are quite different. The *Blastomyces* of Gilchrist is a hyphomycete, and the other a yeast-like fungus. The latter and *Candida albicans* are the only proven yeast-like pathogens. One would think that with the numerous publications on the subject, the fact that these fungi are quite distinct and different from *Blastomyces dermatitidis* would now be very clear. However, in a recently published text, "The Fungi,"<sup>7</sup> which appeared in two volumes, only a part of a page is given over to the discussion of these two diseases, and that under the heading *Cryptococcus histolyticus*. We will quote the final sentence of this brief discussion: "A generalized blastomycosis, manifest as cutaneous abscesses, is caused by the *closely related* *Blastomyces dermatitidis*, also known as *Gilchristia dermatitidis* or *Zymonema dermatitidis*." This sentence alone would seem to justify repeating these well-known facts. Evidently, it is necessary to emphasize again that these diseases are distinct and due to entirely different fungi, and that we should no longer use the term blastomycosis in speaking of Busse and Buschke's disease. It is due to a yeast-like fungus which reproduces by budding, does not form mycelium or ascospores, and which therefore belongs to the group of the cryptococci. The disease which it causes should be called "cryptococcosis."

There follows a discussion of these diseases in detail, with brief mention of the clinical and pathological aspects and stress laid on the laboratory diagnosis and the specific fungus involved in each case.

### *Cryptococcosis*

*History.* The first description of this infection, according to Freeman,<sup>8</sup> was that of Zenker<sup>9</sup> (1861). There seems, however, some doubt about the validity of his case, as there was no culture, and the honor of first describing the disease usually goes to Busse<sup>10</sup> and Buschke.<sup>11</sup> Their case, however, differed somewhat from those that followed, as it was one of the rare cases with cutaneous manifestations. The patient, a woman, had an abscess of the tibia from which was obtained a thick, gelatinous exudate in which the yeast-like organisms were noted. The fungus was obtained in culture and its pathogenicity proved by reinoculation into the patient's skin. The infection became generalized and terminated fatally. Similar cases with involvement of the bones, subcutaneous tissues or, in some instances, the viscera have since been described under the names yeast infection, saccharomycosis, blastomycosis, or torulosis.

In 1906, von Hanseemann,<sup>12</sup> in Germany, reported a yeast infection of the brain with similar gelatinous abscesses but without reference to Busse and Buschke's case. In 1916, Stoddard and Cutler<sup>13</sup> described two cases of their own which they concluded were identical with von Hanseemann's and with three other examples which they found in the literature. They described clearly the clinical and pathological differences between this disease and Gilchrist's blastomycosis and the sharply different characteristics of the causative fungi. They gave the name *Torula histolytica* to this fungus and "Torula infection" to the disease.

Unfortunately, Stoddard and Cutler were misled by Buschke's assertion (later retracted) that his yeast produced endospores, and concluded that his and similar cases were a different disease which they called "true yeast infection." They stated that torula infection might affect other organs than the central nervous system, but never the skin. Cases with subcutaneous or visceral involvement continued to be reported as blastomycosis, or under other names.

In 1934, Lodder<sup>14</sup> and the author,<sup>15</sup> independently, reported studies in which the strain of Busse and Buschke was compared with strains from torula meningitis and found indistinguishable. This showed that, whereas one name, blastomycosis, had been used for several distinct diseases, one disease, cryptococcosis, had been described under several names.

Other reviews and monographs have been published and there is now a vast literature on the subject, but time will not permit a discussion of these. Attention should be called, however, to a book which appeared in 1946 by the Australian workers, Cox and Tolhurst.<sup>16</sup> They list a total of about 120 cases, including 33 from Australia. This book covers every phase of the disease in a most complete and thorough manner and is invaluable to anyone interested in the subject. These authors obtained cultures from 10 cases and state that they believe this to be the greatest number isolated in any one laboratory. In our laboratory at the College of Physicians and Surgeons, we have identified 9 strains, 1 in 1937 and 8 since 1945. This raises the question whether the increase of the disease is actual, or should be attributed to better diagnosis.

*Symptomatology and Treatment.* Cryptococcosis, European blastomycosis, or, as more commonly known, torula meningitis, is a chronic infection most frequently limited to the central nervous system, though it may involve other organs, the skeletal structure, and the skin. The symptoms are primarily those of severe headache, vomiting, and stiff neck, with little or no fever. These are sometimes accompanied by disturbed vision and mental confusion. Diagnosis may then be confused with intracranial tumor or tuberculous meningitis, and may only be confirmed with certainty by demonstrating the presence of the organism either directly or on culture. The respiratory system may be involved, but this is uncommon; and infection of skin and bones is rare. There is a striking absence of inflammatory response in lesions of the brain and meninges. In skin or bone abscesses, a thick, mucoid, blood-streaked pus forms which appears upon examination to be almost a pure culture of the organism, but some granulation tissue

develops about the abscess, and pseudotubercles are described. The central nervous system cases are almost invariably fatal. No form of treatment has proved successful. Spontaneous remissions occur, which make it difficult to evaluate treatment. However, Marshall and Teed<sup>17</sup> report a cure with sulfadiazine, and Keeny, Ajello, and Lankford<sup>18</sup> (1944) found that sulfadiazine was effective *in vitro*, whereas sulfathiazole and sulfamerazine were not. Sulfadiazine was ineffective with one of our patients.

There are conflicting reports as to sensitivity to penicillin, and it may be that there are strain variations. Streptothricin is reported as active *in vitro*, more so than streptomycin. Protoanemonin was shown by Holden *et al.*<sup>19</sup> to inhibit the cryptococcus in a dilution of 1-80,000. When injected into a bone abscess in one of our cases, this antibiotic caused the cavity to become sterile after 4-5 weeks.

Further search of new antibiotics with which to treat this disease is urgently needed.

**Diagnosis.** Serology is of no value in establishing a diagnosis; and the skin test has not been sufficiently studied as yet to be considered reliable, although it has been reported positive in a few cases. Spinal fluid findings may be of some value, as it has usually a low chloride and sugar content and is under increased pressure. But, as emphasized many times, finding *Cryptococcus neoformans* either directly or in culture is the only certain means of diagnosis. It has been demonstrated in, or isolated from spinal fluid, pus, sputum, urine, and blood, the last two having been reported as a source of culture in only very few instances.

The material for examination is placed on a slide with a drop of India ink. In such a preparation, the organism appears as an oval or spherical budding cell with a definite wall surrounded by a clear area, or halo, which constitutes the capsule. Gram stain of fixed smears will serve also to demonstrate the fungus, the cell being gram positive, the capsule faintly pink or unstained. The fungus may be grown out from the material referred to on the usual Sabouraud's medium or blood agar in 4-5 days at 37° C. or in 7-8 days at room temperature.

The cryptococci may be defined, as just stated, as yeast-like fungi of oval, globoid, or spherical shape, reproducing by a process of budding. They do not form spores or produce true mycelium.

**Etiological Agent:**—*Cryptococcus neoformans*

**Nomenclature.** As the name *Torula* is still commonly used in the medical literature for this fungus, a brief resumé of the terminology should be given.

The generic name *Cryptococcus* was first given by Kützing<sup>21</sup> to an organism which he found on moist window panes and which he classified among the algae. His herbarium specimen is extant in sufficiently good condition to prove that his fungus multiplied by budding. Vuillemin<sup>21</sup> adopted the term for pathogenic yeast-like fungi which did not develop ascospores, and gave the name *Cryptococcus hominis* to the Busse-Buschke organism. There seems as yet no cause to abandon its use.

The name *Torula* was first used by Persoon<sup>22</sup> to designate one of the Dematiaceae, a form with short, dark hyphae and chains of globose conidia.

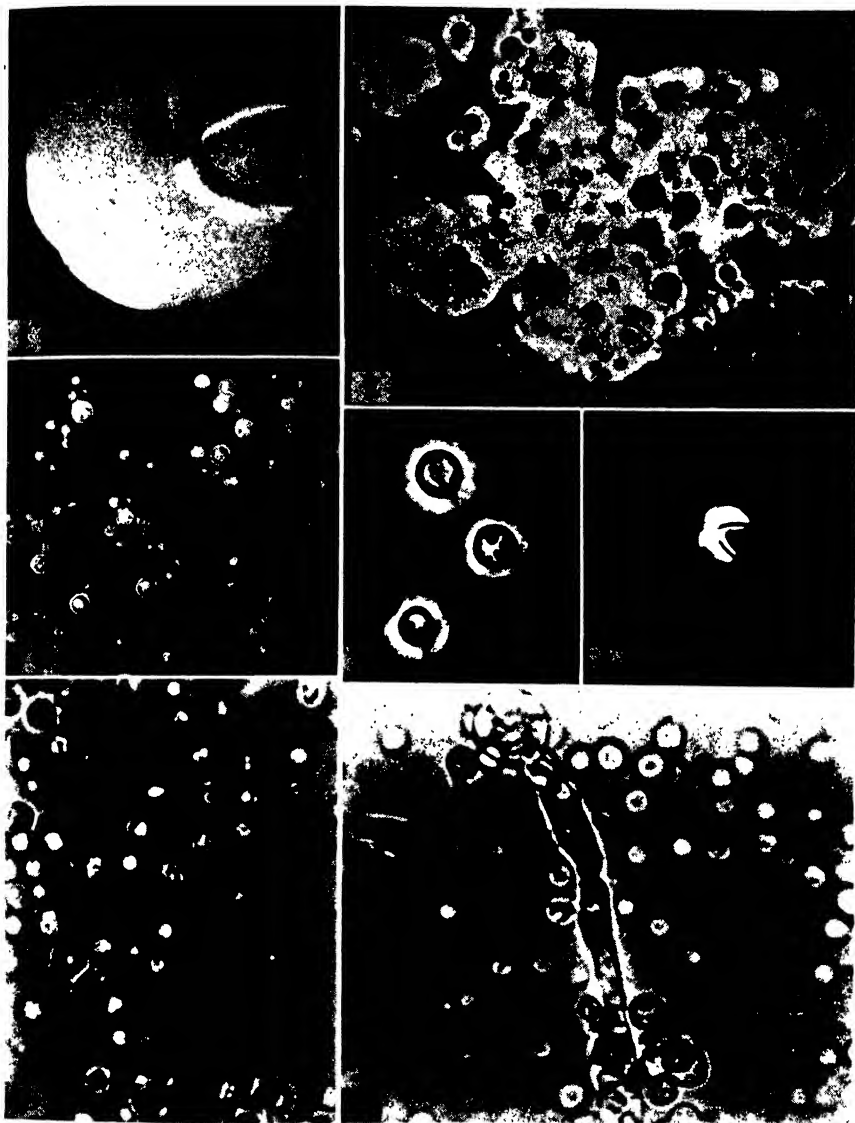


PLATE I. *Cryptococcus neoformans*.

- FIGURE 1. Colony on dextrose agar 1 month.  
 FIGURE 2. Budding cells in rat tissue—showing capsule. ( $\times 400$ ).  
 FIGURE 3. India ink mount of culture showing spherical budding cells surrounded by narrow capsule. ( $\times 160$ ).  
 FIGURE 4. Encapsulated cells from peritoneal cavity of mouse. India ink preparation. ( $\times 400$ ).  
 FIGURE 5. Empty shell-like structure, oval body having escaped.  
 FIGURE 6. Culture mount from cornmeal agar showing thick walled or capsulated, irregularly-shaped cells, containing oval body.  
 FIGURE 7. Culture mount of *C. neoformans* showing pseudomycelium.



been able to produce sera with a titer of 1-160 by treating the cells with acid to remove the capsule before immunizing, and then giving weekly injections for 10 or 12 weeks.<sup>15</sup>

A number of strains from various sources, including that of Busse and Buschke, were found to be antigenically identical. The tests, however, did not serve to separate the *C. hominis* strains from those labelled *T. histolytica*. One pathogenic strain, the so-called *Saccharomyces tumefaciens* of Curtis, was found to be different. These reactions did not definitely separate the pathogens from the non-pathogens, as several of the latter reacted well with *C. neoformans* serum and only feebly with the serum prepared against nonpathogenic strains. For instance, a nonpathogenic strain isolated from the feces of a healthy individual proved to be similar antigenically. It completely absorbed agglutinins from 3 pathogenic sera, but the reverse did not hold true. The pathogenic strains did not react strongly with the nonpathogenic serum.

Cox and Tolhurst<sup>16</sup> report sera with a titer of 1-128 by giving 9 injections at intervals of 5 days of a formalized suspension. Others have reported negative results in their attempts to immunize rabbits.

Further studies are being made with our recently isolated strains in an attempt to produce sera with higher titer and thus render more adequate this method which promises to have value in the understanding of these fungi. Complement fixation tests have so far been reported negative, both with the patient's serum and with serum from immunized rabbits.

*Pathogenicity and Virulence.* The organism is pathogenic for the usual laboratory animals, with rats and mice the most susceptible, rabbits and guinea pigs less so. Virulence does not seem to change on artificial media. The strain purported to be the original of Busse and Buschke is still pathogenic for animals. The virulence does seem to vary with different strains. The animals likewise show individual difference. Of any inoculated group, some will show more resistance than others and survive longer, but according to Cox and Tolhurst,<sup>16</sup> no inoculated mouse escapes the infection. Lesions in the brain, and other tissues similar to those seen in the human, are encountered.

*Epidemiology.* The way in which infection takes place is not clear. The disease occurs sporadically in both man and animals and is met with in all parts of the world. It does not spread from person to person. Organisms similar, except in virulence, for laboratory animals, and in the ability to grow at 37°, have been isolated from the skin and intestinal tract of normal individuals. The *Saccharomyces* isolated from fruit by Sanfelice appears identical. With these exceptions, *C. neoformans* has not been recovered except from lesions. Some mutations may account for the pathogenicity. The acquiring of a capsule may serve to protect the organisms, which then find the tissues a favorable environment for growth.

There is much need for a careful study and comparisons of case histories in the hope that light will be thrown upon the epidemiology of this dreaded disease.

*Blastomycosis*

Gilchrist's disease, or North American blastomycosis, is a chronic disease caused by the hypomycete, *Blastomyces dermatitidis*. There are two forms of the disease, the cutaneous and the systemic. The more common variety is the cutaneous, and this is characterized by granulomatous lesions which may occur on any part of the body. Localized skin lesions may heal completely, but disseminated forms extend slowly until death. Blastomycosis may begin as a pulmonary infection and affect other viscera. Central nervous system lesions may follow a preliminary infection of the skin or may arise from some hidden focus, probably the lungs. The visceral forms are frequently fatal, and the central nervous system cases invariably so.

*History.* The first description of the disease is that of Gilchrist<sup>38</sup> and Gilchrist and Stokes.<sup>6</sup> In the first case, the patient was a man with a lesion on the hand resembling a verrucous tuberculosis. In sections, round and oval refractive bodies were found but no culture was obtained. From the second case, that described two years later with Stokes, Gilchrist obtained a culture which developed mycelium. This was at first called *Oidium*, but later, on the supposition that it was the same as the Busse-Buschke organism, it was named *Blastomyces dermatitidis*. Other cases were reported and a good description of the disease given by Montgomery and Ormsby<sup>39</sup> in 1908. The infection seems to be limited to North America, with cases reported from at least 28 states and the most by far from Chicago. Only two of the proven cases have occurred outside North America, one in Canada and one in England. Martin and Smith,<sup>40</sup> in a review published in 1939, have collected a total of 347 possible cases, with 80 of them proven. Of the others, it is difficult to say in just how many the fungus concerned was *Blastomyces dermatitidis*.

*Symptomatology.* The cutaneous form of the disease apparently results from surface infection and the first lesions are described as pustules. They may appear on the face, hands, or any exposed site. In the neighborhood, new lesions form and fuse with the older ones to form a plaque with a raised border which slopes down to normal skin. The central portion becomes covered with crusts, and miliary abscesses are usually visible near the margin. Larger lesions show some central involution and cover large areas of the trunk in a serpiginous pattern. These lesions are relatively painless. Fever or subjective symptoms are rare.

The systemic variety may, as has been said, follow primary infection of the skin, although, in 50 per cent of the cases, the first symptoms are referable to the respiratory tract. Subcutaneous nodules form and, as the disease progresses, break down and ulcerate, thus resembling the cutaneous nodules of cryptococcosis. Granulomatous nodules may also appear in the lungs, larynx, bones, liver, kidney, spleen, genitals, serous membranes, and central nervous system.

*Treatment.* Iodides and Roentgen therapy are of value in the treatment of the cutaneous disease, but iodides have little effect in the treatment of the systemic infection. Gentian violet inhibits growth of *B. dermatitidis*

in a dilution of 1:500,000 (Sanderson and Smith,<sup>41</sup>) but is of little therapeutic value. Noojin and Callaway<sup>42</sup> tested seven sulfonamides against *B. dermatitidis* *in vitro* and found that sulfonamylamide and sulfadiazine were the most effective. Concentrations necessary to effect the fungus would, however, be too high for clinical use, except perhaps as applied locally to cutaneous lesions. Foster and Woodruff<sup>43</sup> report that *Blastomyces dermatitidis* is unaffected by penicillin, even in doses as high as 30 oxford units per cc., but streptothricin inhibits the fungus in concentrations comparing favorably with those required for the inhibition of *Escherichia coli*. Keeney, Ajello, and Lankford<sup>18</sup> also reported negative results with penicillin. Tishman<sup>44</sup> reports a new form of therapy which may offer hope. After iodides and sulfa drugs proved ineffective, ether was applied locally to the skin lesions and used to irrigate the osteomyelitic infection. In addition, it was mixed with an equal amount of oil and given rectally after soapsuds enema. Treatment was begun in October and the patient was discharged as cured in February. The patient died three years later from other causes and, at autopsy, gave no evidence of infection with *Blastomyces*, nor any evidence of harmful effect of the drug. The effectiveness of ether has not yet been confirmed by other observers.

**Diagnosis.** As with other fungus diseases, the diagnosis can only be made definite by finding the characteristic organism either upon direct examination or in culture. Skin tests may be a real aid, as in this case it is a specific reaction similar to a positive tuberculin reaction. The reaction appears in 12-23 hours, reaches a maximum in 2-4 days. In very allergic patients, the reaction may proceed to a sterile abscess. A negative or weak reaction may result in patients in the terminal stages of the disease. On the other hand, positive reactions have not been noted in patients without blastomycosis. Martin<sup>45</sup> believes that a skin test should always be made before starting treatment with iodides. If the reaction is less than 1 cm. in diameter, it is safe to administer potassium iodide, but, if 1 cm. or more, the reaction indicates that the patient is hypersensitive and should be desensitized before iodine therapy is started. This is done by the subcutaneous injection of gradually increasing amounts of vaccine, beginning with saline dilutions. Iodides may be administered after 2 weeks of vaccine injections. Surgery also is of value in treatment, and X-ray therapy has proved helpful in clearing cutaneous lesions, provided the hypersensitivity of the patient has been reduced by vaccine therapy.

**Etiological Agent:** *Blastomyces dermatitidis* (Gilchrist and Stokes, 1898)

**Nomenclature.** Many names have appeared in the literature to designate this fungus. Some, such as *Oidium*, *Zymonema*, *Acladium*, *Aleurisma*, and *Endomyces*, express the authors' belief as to the botanical classification of the organism. Others were given because the authors failed to recognize the identity of their strain with the species described by Gilchrist. Ota,<sup>46</sup> as a result of his studies, concluded that a strain isolated by Gammel, and named *Glebospora gammeli* by Pollacci and Nannizzi,<sup>47</sup> and two strains of *Blastomyces dermatitidis* obtained from Weidman were identical, but added to the confusion by naming the organism *Acladium gammeli*, and later with Kawatsure<sup>48</sup> renaming it *Aleurisma tulanense*. Castellani<sup>3</sup> had pro-

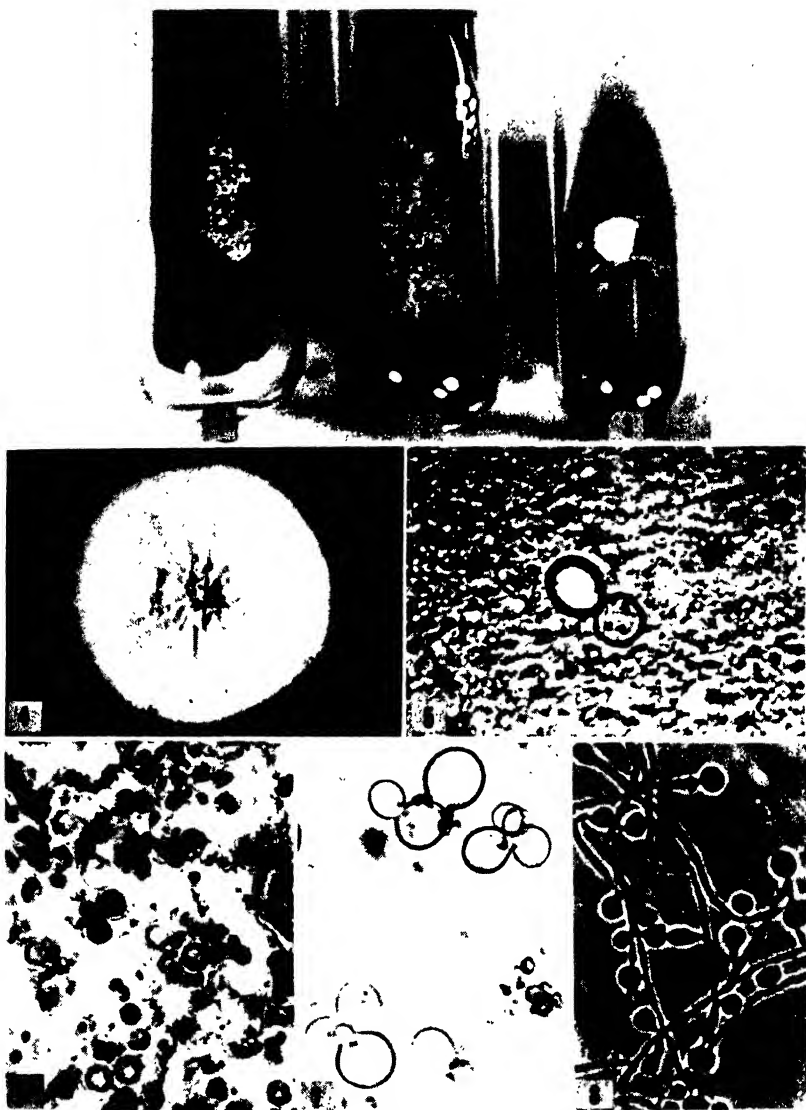


PLATE II. *Blastomyces dermatitidis*.

FIGURES 1, 2, and 3. Cultures at 37° C. (1) Yeast-like, on blood agar. (2) and (3) Intermediate stages on blood agar.

FIGURE 4. White, downy colony on Sabouraud's dextrose at room temperature.

FIGURE 5. Budding cells in pus, direct from lesion. (× 400).

FIGURE 6. Budding cells in lung of dog. (× 400.)

FIGURE 7. Culture at 37° C. on blood agar showing yeast-like cells. (× 400.)

FIGURE 8. Culture at 37° C. showing mycelium and conidia. (× 400.)

posed the new genus *Blastomycoides* for the fungi causing American Blastomycosis. He made the mistake of including *Blastomycoides immilis* in the genus.

In 1934, in a comparative study of the fungi of blastomycosis and coccidioidal granuloma,<sup>49</sup> I showed that *Glenospora gammeli* (Pollacci and Nannizzi, 1927), *Blastomycoides* (monosporium) *tulaneuse* (Castellani, 1928), *Endomyces capsulatus* (Newbridge, Dodge, and Ayers, 1929), *Endomyces capsulatus*, variety *Isabellinus* (Moore, 1933), and *Endomyces dermatitidis* (Moore, 1933) were identical with *Blastomyces dermatitidis*. Ciferri and Redaelli<sup>50</sup> also showed these strains to be identical and proposed the name *Gilchristis dermatitidis*. Conant<sup>8</sup> once more confirmed these authors and compared many of these same strains with several strains of *Plastomyces dermatitidis* (Gilchrist and Stokes, 1898) isolated from cases of blastomycosis at Duke. Dodge<sup>51</sup> applied the name *Zymonema* (Buermann and Gougerot, 1909) to this fungus, giving as the type species, *Endomyces dermatitidis* and placing it in the lower ascomycetes, following Moore's<sup>52</sup> report of asci in this strain. The development of asci has not been confirmed, and there seems as yet no valid reason for replacing the name used by Gilchrist, *Blastomyces dermatitidis*.

*Characteristics: In Tissue.* The fungus appears in the patient's lesion or in those of inoculated animals as round or oval cells usually 8-10  $\mu$  in diameter, though often as large as 20  $\mu$ . They usually produce one bud at a time and the cells are highly granular. In sections, the wall is sharply differentiated from the contents, which are somewhat shrunken by fixation, thus pulling away from the wall and leaving a space. This, I believe, has led to the wall's being referred to as a capsule. There is, however, no capsular substance comparable to that of *Cryptococcus neoformans*. In tissue, the organisms may be found in large masses, but more often mingled with leucocytes in an abscess, scattered through the granulation tissue or phagocytes by giant cells.

*Characteristics: In Culture.* This fungus assumes a variety of forms, depending on the medium and incubation temperature. It grows well on Sabouraud's media and is isolated most easily from the pus from miliary abscesses. It takes from 10 days to 2 weeks to appear. If the first cultures are incubated at 37° C. and on blood agar, a brownish, waxy to smooth growth occurs, which Henrici<sup>53</sup> referred to as the "mealy" type. In this type of growth, the fungus maintains the yeast morphology with only occasional elongated forms, indicating a transitional stage. Some cultures become covered with tufts or spiny elevations which resemble coraemia. This is Henrici's "prickly" type. As soon as the cultures are brought to room temperature, however, the yeast-like growth is lost, and an extensive, branching mycelium forms. On Sabouraud's maltose or dextrose medium, the fungus grows, slowly forming a downy to fluffy colony, at first white but later deep cream or tan. In giant colonies, the central zones may show radial folds and often concentric rings. The colony often resembles the *Trichophyton*s. Ricketts<sup>54</sup> recognized these three types of growth.

In these downy colonies, rather characteristic conidia, first described by Gilchrist, are borne on the sides of the hyphae or on the tips of short lateral branches. They appear first as knoblike projections, which gradually enlarge. At first, the wall is thin and the contents are more or less homogeneous. Later, the walls thicken and the contents become more granular, giving somewhat the appearance of chlamydospores. These cells may become free and appear to bud, resembling the cells observed in tissue. These conidia are found regularly in the downy or fluffy colonies. All transitional forms, from yeast to fluffy, may be seen.

*Biological.* As reported by Levine and Ordal,<sup>55</sup> *B. dermatitidis* does not seem to require accessory growth factors. The growth on a medium of salts, glucose, and ammonium sulfate is never equal to that on a more complete peptone glucose medium. This would indicate a stimulating action of amino acids. On a complete medium, the organism grew well at all pH levels except 5.5 at room temperature, but at 37° no growth occurred at pH 4.5 or below. On a deficient medium, the quantity of growth increased as pH concentration decreased. Also, in general, the higher the temperature is, the greater the amount of growth. The effect of temperature on the form of growth has already been mentioned. The tendency to form mycelium decreases as the hydrogen ion concentration and temperature increases.

*Serology.* Sera of patients with extensive lesions will fix complement with suspensions or extracts of *B. dermatitidis*. No cross reaction with extracts of other fungi occur, and the reactions are always negative in the absence of blastomycosis.

In mild infections, that is, in patients with the localized cutaneous forms, no fixation takes place. According to Martin,<sup>45</sup> a fatal outcome is to be expected in patients with a high antibody titer and a negative or slightly positive skin test. Prognosis is best in the hypersensitive patient without complement-fixing antibodies in his serum.

*Pathogenicity and Virulence.* Pathogenicity, as in the case of the cryptococcus, seems to be a fixed character, for, after years on artificial media, this fungus will reproduce the disease in animals. Of the laboratory animals, dogs and monkeys seem most susceptible. Heavy subcutaneous inoculation results in a local suppurative granuloma, with metastatic lesions in the lungs and other viscera. Spring<sup>56</sup> found mice more susceptible than guinea pigs and rabbits. In mice, typical lesions should develop in 3 weeks, with lesions in liver, spleen, lungs, and lymph nodes. Examination of the fresh material from these lesions or from the peritoneal fluid will show the typical parasitic stage. Baker<sup>57</sup> attempted to enhance the virulence for mice by repeated transfer, but failed to do so.

*Epidemiology.* The source of the infection is unknown. Stober<sup>58</sup> observed, in rotting wood from the rooms where patients with blastomycosis had lived, fungi which resembled the parasite, but was unable to prove their identity. Infection, however, probably is from some exogenous source. The disease has been known to occur spontaneously in animals. Foshay and Madden<sup>59</sup> reported the first spontaneous case in a dog. The organism

was obtained on culture, and the serum from the dog was completely fixed with typical *Blastomyces* strains.

### Summary

In this report, attention has been called to the fact that the disease described by Busse and Buschke, in Europe in 1894, and those meningeal infections later described by Von Hansemann, Stoddard and Cutler, and others are really one and the same disease, caused by a yeast-like fungus, *Cryptococcus neoformans*. Also, it has been indicated that North American blastomycosis is an entirely different disease, caused by a mycelium-producing fungus, *Blastomyces dermatitidis*. A detailed consideration of these two fungi has been given and the correct terminology discussed. Once more, the suggestion is made that the term *Blastomyces* be discontinued, except as it pertains to Gilchrist's disease, and that *Blastomyces dermatitidis* be made a *nomen conservandum* for the fungus involved. Likewise, it is suggested that the name cryptococcosis replace torulosis or torula meningitis for the meningeal cases, and that *Cryptococcus neoformans* also become a *nomen conservandum*.

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## THE NUTRITIONAL REQUIREMENTS OF THE FAVIFORM TRICHOPHYTONS\*

By Lucille Katharine Georg

*Departments of Dermatology and Bacteriology, Columbia University College of Physicians and Surgeons, New York, N.Y.*

In a recent report, "Suppurative Ringworm Contracted from Cattle,"<sup>1</sup> a series of twenty-three cases of deep, suppurative ringworm have been described. The cases appeared during a three-year period among a community of farmers and their families in central Pennsylvania. These unusually severe ringworm infections occurred in order of frequency on the neck, face, wrists, forearms, and in the occipital region of the scalp. The cases were divided into three groups: the sycosis parasitica type, or suppurative ringworm of the bearded areas of the face and neck; the kerion type found in the scalp; and agminate folliculitis, or suppurative lesions of the glabrous skin. In all instances, there was a history of recent contact with cattle, and, in all but five cases, the patients had observed ringworm lesions on their cattle. The lesions, their clinical course, and their treatment, have been described in detail.

Cultural studies indicated that fourteen of these cases had been caused by the large-spored faviform trichophytons of the *album*, *discoides*, and *ochraceum* varieties, and four cases by the common *T. mentagrophytes*. In the remaining five cases in this series, no parasitized hairs were found and no cultures were obtained. The nature of the lesions, however, the absence of bacterial infection, and the history of contact with cattle infected with ringworm, suggest that these cases also were caused by ringworm contracted from cattle.

The faviform trichophytons are characterized by their large-spored, ectothrix parasitism of the hair, and by the extremely slow-growing, non-sporulating type of growth which they produce on the usual laboratory mediums. On Sabouraud's dextrose or maltose mediums, the cultures are usually of a glabrous type varying from moist, heaped-up, waxy, cerebriform colonies to colonies covered with a very fine short white down, which appears irregularly on their often highly convoluted surfaces. Such colonies are composed of an irregularly branched mycelium similar to that characteristically seen in cultures of *Trichophyton schoenleini*, the causative organism of favus. Chlamydospores may be present in large numbers, but microconidia and macroconidia are rarely produced on these mediums.

The poor growth of the faviform trichophytons on the usual mediums makes their isolation from ringworm lesions as well as the maintenance of stock cultures extremely difficult. Their inability to produce spores on the usual mediums has led to great confusion in their classification. However, when enriched or natural mediums are used, microconidia and macroconidia typical of the *Trichophyton* group have been produced.<sup>2, 3</sup>

In our recent experience in the isolation of faviform trichophytons from

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ringworm lesions, it was found that, by the use of a medium rich in peptones such as heart infusion-tryptose agar (blood agar base, "Difco"), not only were the chances of isolation greatly improved, but continued good growth with production of microconidia and macroconidia could be obtained in most instances. This was particularly true when thiamine was added to the medium, and it was recommended that heart infusion-tryptose agar with added thiamine (0.1 mg. per 100 cc.) be used for the isolation, study, and maintenance of these cultures.

These findings suggest that these organisms are deficient, that is, they are unable to synthesize (at least in sufficient quantities) certain growth factors necessary for the initiation and maintenance of growth and for the production of spores. That thiamine may be one of these factors is indicated by the production of rapid-growing, vigorous, fluffy colonies with many spores when this vitamin is added to a rich peptone medium. The possibility of other growth factors in the heart infusion-tryptose medium is also to be considered.

It is the purpose of this paper to study this group of "deficient organisms" in order that a better understanding of their growth characteristics and requirements may be obtained.

*History.* A trichophyton of faviform type was first observed by Sabouraud in 1893.<sup>4</sup> He isolated the organism from a child who had a flat lesion on the temple which was suppurating at many points from gummy, cracked crusts. The culture developed with extreme slowness and showed characteristics analogous to those of *Achorion faveus*, the causative organism of favus, now known as *Trichophyton schoenleini*. Preparations of the diseased hairs, however, did not reveal an endothrix parasitism, as seen in favic hairs, but they did show an ectothrix fungus with large spores in mosaic and occasional chains of spores lying along the side of the hair. The radical part of the hair was filled with branching mycelium. The spores varied in size from 5 to 8 microns.

E. Bodin, who studied this organism, was also able to isolate several similar cultures from lesions on horses, a donkey, and a calf. Bodin<sup>5</sup> has furnished a description of a species which he called "*Trichophyton faviforme du veau*" (it was later named *T. verrucosum* [1902]): "On glucose agar the culture forms a little cake in a month's time. Part is submerged in the agar and the surface is irregular, verrucose, and grey. It may show a central accumulation." In the epidemic among horses reported by Bodin, the hairs showed that the parasite was an ectothrix fungus. He also observed the infection in nine persons and described the human lesions as a "thick kerion in the form of a cupola riddled with suppurating follicles."

In 1908, Sabouraud<sup>6</sup> described two species in this group. One, which he called *Trichophyton ochraceum*, corresponds well to the *T. verrucosum* described by Bodin except that it is a brilliant yellow ochre. The second species, *T. album* is difficult to distinguish from *T. schoenleini*. Sabouraud described it on 3 per cent peptone medium as a glabrous, waxy colony of spongy surface and vermicellular appearance. On maltose agar, it is heaped-up and shows an umbilicate center and sloping sides which have many

irregular folds. The texture is tough and rubbery. The cultures were extremely slow-growing and mediocre in development compared to favus cultures. He described lesions of four types caused by these organisms: (1) typical kerion lesions with considerable subcutaneous suppuration; (2) plaques resembling seborrheic eczema; (3) typical ringworm lesions of the annular type with cleared center and vesicular outer border; and (4) young lesions which were dry and leathery in appearance.

In 1910, Sabouraud<sup>6</sup> described a fourth species, *Trichophyton discoides*, which he stated was somewhat similar to *T. album*, except that its growth was almost a perfect disc with a flat surface and often a central knob. The whole growth had a light tan to brown color with a moist surface. The cultures may develop a fine, short, white down. This organism was habitually found in cattle and produced lesions of the kerion type in man.

Sabouraud, who used only sugar mediums with small amounts of peptones, observed only the slow-growing, glabrous, faviform colonies described above. He did not feel, however, that the appearance of the cultures was of enough importance to classify the organisms among the *Achorions*, and, although unable to show any structures characteristic of trichophytons, he placed them in the *Trichophyton* group on the basis of the large-spored ectothrix parasitism of the hair and the tinca-like lesions produced in man as well as in the experimental animal.

Many workers studied the organisms of the faviform group in an attempt to obtain spore-bearing structures which would more clearly identify them as members of the *Trichophyton* group. The introduction of the "natural mediums of polysaccharide base" by Langeron and Milochevitch,<sup>7</sup> greatly aided this study. On these mediums, which consisted of whole grains of wheat, barley, corn, and oats, it was found that the faviform trichophytons developed quite rapidly and were very different in appearance from the glabrous colonies obtained on the sugar mediums. The growth was of a white, velvety to fluffy character similar to the growth of the more common trichophytons. Microscopic examination revealed microconidia characteristically borne by the hyphae in clusters (*en grappe*) and laterally (*en thyse*). Also, rudimentary spirals and macroconidia typical of the *Trichophyton* group were demonstrated.

In 1934, Lebasque<sup>8</sup> carefully reviewed the faviform group and was able, by the use of the polysaccharide mediums, to complete the morphological studies of the species already known, as well as three new species. Of the large number of species which had been described by various workers on the basis of the gross appearance of the colonies, Lebasque accepted only six as having characteristics sufficiently distinct to be considered as different species: *T. verrucosum* (Bodin, 1902), *T. ochraceum* (Sabouraud, 1902), *T. album* (Sabouraud, 1909), *T. discoides* (Sabouraud, 1910), *T. equinum* (Geddoelst, 1902), and *T. caballinum* (Neveu-Lemaire, 1921). He also described three new species: *T. bullosum*, *T. villosum*, and *T. papillosum*. For all these species, Lebasque was able to obtain cultures which showed all the macroscopic and microscopic characteristics typical of the *Trichophyton* group. He described the parasitism of the hair by these species as endo-ectothrix, because of the vigorous development of the flexuous,

dichotomously dividing mycelium in the interior of the hair. This picture, however, except for the larger size of the spores, is not different from that occasionally seen in microsporum infections, where the interior of the hair may be filled with branching mycelium. The presence of mycelial filaments in the interior of the hair, as described by Baudet<sup>8</sup> and often seen by us in examination of hairs from cattle as well as from patients, probably is indicative only of the early stages of invasion by the fungus. There seems to be no valid reason for calling the parasitism endo-ectothrix.

One of the species studied by Lebasque, *Trichophyton equinum* (Geddoelst), presented a downy to fluffy culture on the classic "proof medium." However, spores were not obtained until the culture was grown on the natural carbohydrate mediums. Lebasque did not feel that there should be a distinction between the glabrous and downy members of the group as had been suggested by Castellani and Chalmers.<sup>9</sup>

That the faviform trichophytons may be vitamin-deficient organisms was clearly shown by Robbins, Mackinnon, and Ma<sup>10</sup> in their study of the Mackinnon strain of *T. discoides* isolated in Uruguay. They demonstrated that this strain required three vitamins for growth: pyridoxine, i-inositol, and molecular thiamine. They were able to produce growth on a basal medium prepared with purified agar, recrystallized asparagine, dextrose, phosphate buffers, and inorganic salts only after these three vitamins had been added. The thiamine could not be substituted for by an equal mixture of thiazole and pyrimidine.

Burkholder and Moyer,<sup>11</sup> have shown that a strain which they call *Trichophyton faviforme* has a deficiency for thiamine and inositol, and that its growth is stimulated by media containing liver and peptones.

Schopfer and Blumer,<sup>12</sup> in their study of the growth requirements of a strain of *T. album* which they isolated, have shown that this particular strain, although able to grow to some extent on a basal medium without the addition of any vitamins, produced a more rapid growth when biotin was added. This was particularly true when a nitrogen source such as ammonium citrate was used in place of asparagine or amino acids. The action of the biotin was shown to be dependent on the physiological age of the culture. They also demonstrated that, in an unbuffered nutrient medium, thiamine, inositol, and pyridoxine clearly furthered the development when asparagine was used as a nitrogen source. Here it was found that an equal mixture of thiazole and pyrimidine would substitute for the thiamine. They obtained best growth when all four of the stimulating substances, biotin, thiamine, inositol, and pyridoxine, were added to a 1 per cent asparagine unbuffered medium at pH 6.0, in which medium they obtained a mycelial weight three times greater than in control flasks.

In contrast to these studies, *T. schoenleini* has never been shown to be stimulated by vitamins. That this is also a deficient organism, however, has been shown by the following findings. (1) Whole grain mediums stimulate the production of rapidly growing, heavily powdery or downy cultures with numerous microconidia. This is in contrast to the usual slow-growing, glabrous cultures obtained on the routine sugar mediums, which rarely

show any spores. (2) Catanei<sup>13</sup> observed that cultures contaminated by bacteria, staphylococci, and certain bacilli, present a powdery or downy aspect in contrast to the glabrous colonies of pure isolates. This has been shown to be due to a water-soluble, heat-stable substance produced by the growth of the bacteria.

### *Experimental*

#### *Part I. Cultural and Morphologic Studies of Recently Isolated Faviform Trichophytons*

Eight strains of faviform trichophytons recently isolated from suppurative ringworm lesions<sup>1</sup> were selected for cultural and morphologic studies. Pure strains were obtained by isolating six single spores from each strain (according to the method described by Georg<sup>14</sup>). Cultures were made on the following mediums: (1) simple sugar mediums—Sabouraud's dextrose and maltose agar ("Difco") and Sabouraud's maltose "proof medium" prepared with crude maltose and French peptone according to the method described by Sabouraud<sup>6</sup>; (2) whole grains—moist rice and barley prepared according to the method of Langeron and Milochevitch<sup>7</sup>; and (3) enriched mediums—dextrose and maltose agar enriched with yeast extract, liver extract, beef heart infusion, Bacto-tryptose, and citrated human blood. Cultures were grown both at room temperature and at 37°C., on moist and dry mediums of pH varying from 5.0 to 7.4.

##### *A. Cultural Characteristics on the Simple Sugar Mediums*

*1. Gross Appearance.* On Sabouraud's dextrose and maltose agar ("Difco") the different strains produced colonies which showed considerable variation from one another even on the same medium. These ranged from completely glabrous, moist, heaped, cerebriform colonies the color of wax to irregularly folded or completely flat, disc-shaped colonies covered with a short white down, and glabrous, highly verrucose colonies of a bright yellow ochre color.

On first isolation or early transplantation to these mediums, all colonies tended to be glabrous and of a tough, leathery consistency. After prolonged cultivation and repeated transplants, however, the large majority developed a fine white powder at the base of their acuminate structure or in the crevasses of their folds. Still other isolates, which presented a flatter colony, often with a central umbo and a scalloped or irregular border, developed a short white down which covered the entire colony. A third type of colony, developed by two of the strains, began as a moist, colorless, skin-like growth over the surface of the medium. This later developed glabrous projecting buttons which were a bright yellow ochre. Often the whole surface of the colony became highly verrucose and bright yellow ochre. These cultures usually developed some fine white powder at the periphery and in the center of the colony which, when dense, gave a powdery gray central area and border to the otherwise yellow ochre to deep orange colonies (FIGURE 1).

In general, growth on these mediums was very slow and meager at room

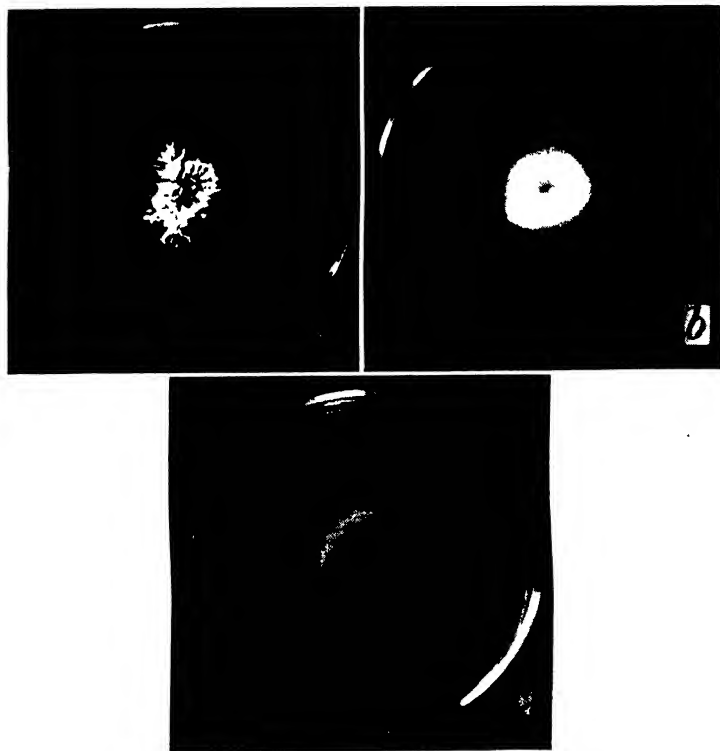


FIGURE 1. *Trichophyton faviforme*. Sabouraud's dextrose agar ("Difco") 40 days: (a) variety *album*; (b) variety *discoides*; (c) variety *ochraceum*.



FIGURE 2. *Trichophyton faviforme*—Sabouraud's dextrose agar ("Difco") Characteristic morphology showing irregular, branched, "faviform" mycelium with numerous chlamydospores. 400X

temperature. At 37°C., the rate of growth was considerably accelerated, but, even under these conditions, the growth did not usually appear until the fifth to seventh day. On the average, colonies grown at room temperature approximated 30 millimeters in diameter after 6 weeks of growth. They were, however, often five to ten millimeters in height in the center. The optimum pH was found to lie between 6.8 and 7.2.

In order to identify these colonial types more clearly with those described by Sabouraud and other workers, the cultures were planted on Sabouraud's maltose "proof medium." On this medium, growth was more rapid and colonies attained a greater size. In general, the colonies were more heaped and folded and tended to develop more powder and down than on the "Difco" mediums. The colonial types were the same, however.

2. *Microscopic Appearance.* Microscopic examination of the cultures developed on Sabouraud's maltose and dextrose agars, both the "Difco" product and the original "proof medium," revealed an irregular branched mycelium with an abundance of intercalary and terminal chlamydospores (FIGURE 2). The appearance, in some cases, was very similar to the "favic chandeliers" produced by *T. schoenleini*. In other cases, the mycelium was less irregular and only produced occasional clubbed ends and intercalary chlamydospores. Reproductive spores, microconidia and macroconidia, were characteristically absent.

3. *Variation.* In general, three types of colonies predominated, which corresponded rather well with the three species described by Sabouraud as *T. album*, *T. discoides*, and *T. ochraceum*.<sup>6</sup> After a series of transplants over a period of several months, however, it soon became evident that these colonial forms were not stable and that a single culture could produce all three of these colonial varieties. In order to determine the nature and frequency of these changes, a series of studies was made using several single-spore cultures from each strain and transplanting serially on Sabouraud's dextrose agar and wort agar ("Difco").

Each strain tended to maintain a rather characteristic colonial morphology which clearly differentiated it as a variety. However, all the variations described above were found in six of the strains studied. A parent culture might be flat and covered with a fine white down, while the subculture would develop into a completely glabrous, heaped cerebriform colony, or a glabrous, flat but highly verrucose colony of the yellow ochre variety. Also, a single culture on wort agar which started as a completely glabrous colony might, in the course of weeks, develop a heavy white down, around the edge of the colony, in isolated tufts, or over the entire surface. That this was not an instance of the development of pleomorphic growth commonly seen in some trichophyton cultures was shown by the fact that these fluffy areas were not composed of sterile hyphae, but were, in fact, rich in microconidia. The fluffy to glabrous change was also common, particularly on the wort agar, and a third change back again to the fluffy state was observed in several cultures (FIGURES 3 and 4). Some strains presented both the heaped-glabrous and the flat-downy type variation in a single colony (FIGURE 5).



Variation in the faviform trichophytons was first suggested by Cazalbou in 1913<sup>15</sup> when he described a species which he called *Trichophyton singulare*, which had two cultural states, a glabrous cerebriform colony and a downy flat disc-shaped colony. The cultures were found to be reversible. In 1938, Gammel and Work<sup>16</sup> described a case of sycosis parasitica due to a faviform trichophyton which they designated as *Favotrichophyton album*,

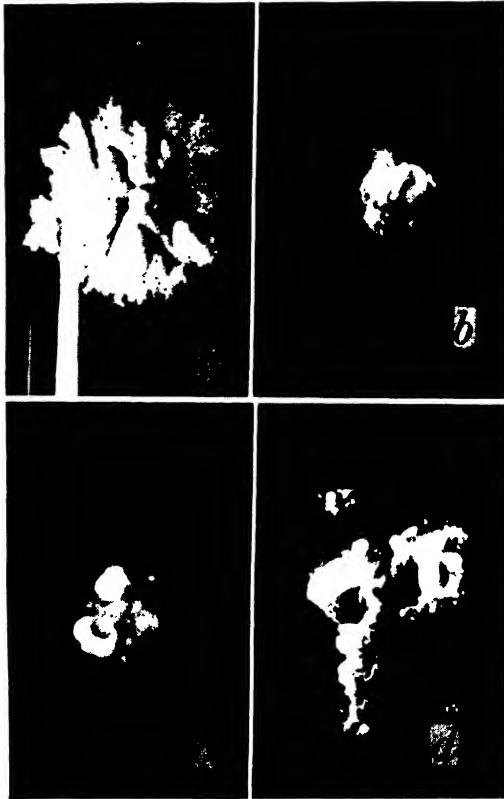


FIGURE 3. *Trichophyton faviforme* (var. *album*)—variation on wort agar: (a) original waxy, heaped, glabrous colony on wort agar; (b) one of many transplants from (a) to fresh wort agar—here the colony is still glabrous, but some of the raised knobs are a bright yellow ochre; (c) later stage of (b) colony begins to show areas of fluffy spore-bearing growth; (d) later stage of (c)—colony shows three types of growth: waxy, glabrous areas; very moist, deep orange areas; and white, fluffy spore-bearing growth.

variety *singulare*. After several transplants on liver infusion agar, they obtained two distinct types of growth, a cerebriform type and a less well-differentiated discoid type.

These findings, as well as our data obtained with single spore cultures, seem to indicate that *T. album*, *T. discoides*, and *T. ochraceum* are not morphologically distinct and suggest that they are variants of a single species. We propose that they be classified as *T. faviforme* (varieties: *album*, *discoides*, and *ochraceum*).

*B. Growth on Whole Grains*

1. *Gross Appearance.* On the natural polysaccharide mediums, moist rice and barley grains, all of the strains grew very well, covering the grains with a felt-like growth. By the tenth day, those cultures which produced some down on the simple sugar mediums showed an aerial mycelium which

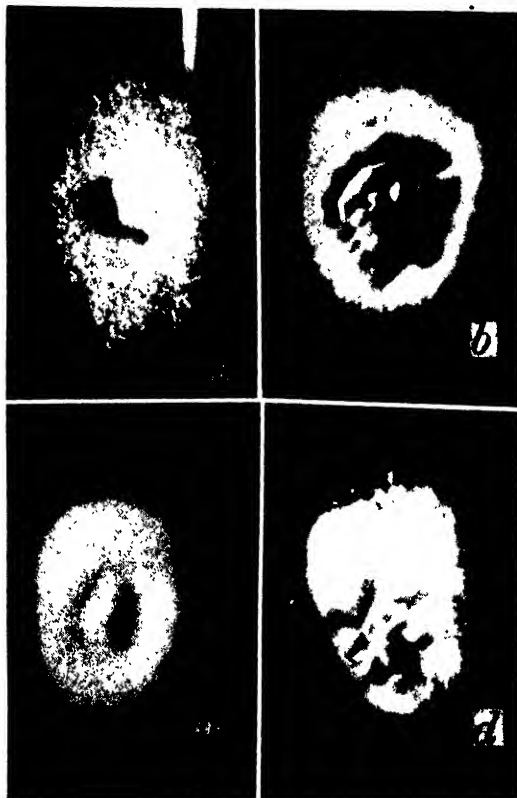


FIGURE 4. *Trichophyton faviforme* (var. *discoides*)—variation on wort agar; (a) original flat colony, with very small amount of short, white down, on wort agar; (b) one of many transplants from (a) to fresh wort agar—here heaping-up of the colony and production of furrows is noted—edge of colony shows beginning of fluffy variation; (c) later stage of (b)—colony is now completely covered with white, fluffy spore-bearing growth; (d) later stage of (c)—the colony became completely glabrous, but now shows a tertiary change with development again of a fluffy mycelium in some areas.

extended from the kernels and was visibly erect. In some cultures, true fluffy growth appeared.

2. *Microscopic Appearance.* The mycelium is well developed in 12 to 15 days and consists of a regular branching, flexuous mycelium with occasional chlamydospores. The aerial growth contains numerous conidia which are seen scattered along the mycelium (*en thyrses*) as well as in terminal pine tree-like clusters (*en grappe*) (FIGURES 6 and 7). These microconidia are not pediculated and break off squarely from their point of attachment

to the mycelium on which they are implanted perpendicularly. They are easily detached and are sprinkled all through the preparation. Their size varies from 1.5 to 2 microns by 3 to 4 microns. Although there is considerable variation in size and shape, in general they tend to be more slender

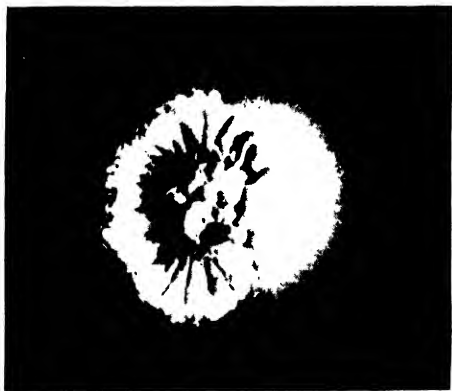


FIGURE 5. Colony showing both album and discoid variation. Developed on Sabouraud's dextrose agar from a single spore culture originally of the album variety.



FIGURE 6. *Trichophyton faviforme*—from growth on rice grains. Microconidia "en thyrsé." 400X

and elongated than the typical pear-shaped or round conidia of the gypseum group.

Macroconidia are also found in the downy to fluffy aerial mycelium appearing from the tenth to thirtieth day. These range from 20 to 30 microns



FIGURE 7 (top). *Trichophyton faviforme*—from growth on rice grains. Microconidia "en grappe." 400X  
FIGURE 8 (bottom). *Trichophyton faviforme*—from growth on rice grains. Macroconidia. 400X

by 5 to 8 microns and have 2 to 8 sections. They occur either terminally or laterally, usually singly at the end of a mycelial branch. Some are short and bulb-like, others show long pointed ends, and others are irregularly swollen and distorted (FIGURE 8).

*C. Growth on Enriched Mediums*

Yeast and liver extracts both had a definite stimulating effect on the growth rate of the organisms when added to the simple sugar mediums. On such enriched mediums, all the strains grew rapidly, producing heavy, compact colonies covered with a white, downy to fluffy aerial mycelium which contained microconidia and occasional macroconidia. The amount

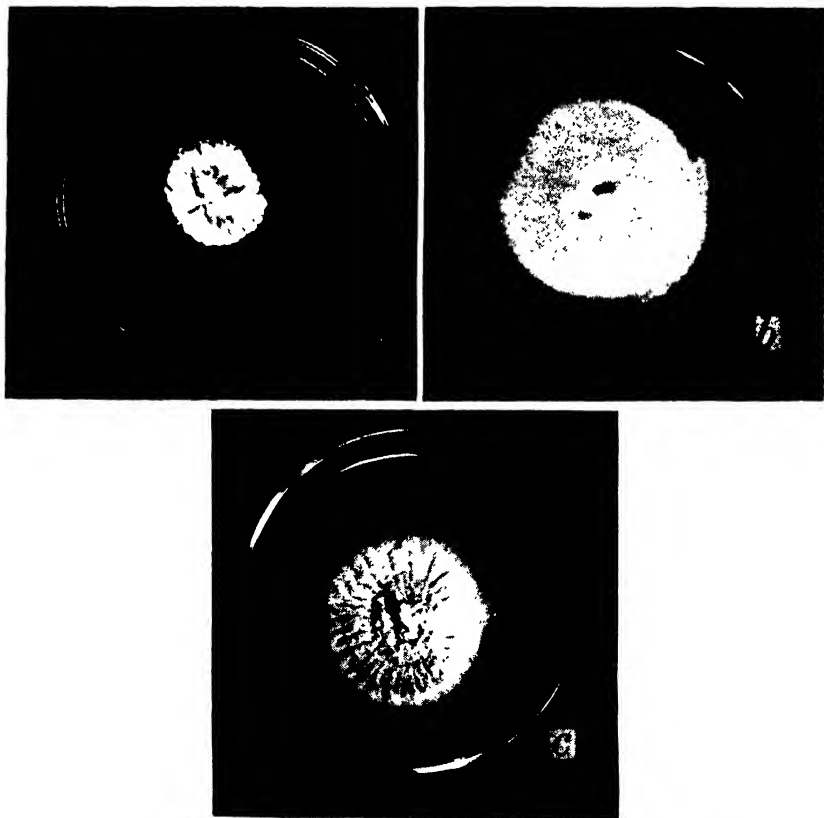


FIGURE 9. *Trichophyton faviforme*—heart infusion-tryptose agar plus thiamine (0.1 mg. per 100 cc.). 40 days: (a) variety *album*; (b) variety *discoides*; (c) variety *ochraceum*.

of aerial growth depended on the strain as well as the amount of the extract added.

Additions of beef heart infusion, Bacto-tryptose, and citrated human blood were studied in view of the fact that, in the original isolation work, blood agar plates prepared with beef heart infusion and Bacto-tryptose (blood agar base, "Difco") had been used and found far superior to Sabouraud's dextrose agar. It was at first thought that the whole blood added to this medium was the stimulating factor. However, no difference could be detected in the amount of growth produced on this medium whether blood

had been added or not. Both the beef heart infusion and the Bacto-tryptose were found to have stimulating effects when added to the simple sugar mediums. Either of these substances, added in amounts comparable to that found in the complete medium, caused the development of rapidly-growing, vigorous colonies which often reached a diameter of 40 to 60 mm. in 4 weeks and were covered with a white powder or a short, erect white down. Addition of thiamine (0.1 mg. per 100 cc.) to the heart infusion-tryptose medium further increased the rate of the growth and produced more aerial mycelium, which was, in most cases, filled with large numbers of microconidia and occasional macroconidia (FIGURE 9).

## *Part II. Studies of the Vitamin Requirements of the Faviform Trichophytons.*

A study was made to determine the vitamin requirements of sixteen recently isolated strains of faviform trichophytons as well as of four stock cultures: *T. album*, *T. discoides*, and *T. ochraceum*, obtained from the Central Bureau for Fungus Cultures, Delft, Holland, and a strain of *T. discoides* isolated in Uruguay, kindly furnished by Dr. J. Mackinnon. The vitamin requirements of this last strain have been studied and reported by Robbins, Mackinnon, and Ma.<sup>10</sup> Synthetic, vitamin-free mediums were used. A basal broth was prepared as follows: a mixture of 2.0 gm. asparagine (recrystallized 3 times), 0.1 gm.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 gm. dextrose (C.P.), and 100 cc. Sorenson's phosphate mixture (M/15  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  at pH 7.0) is made to a liter with distilled water and sterilized. This medium, designated as "Basal Broth A," with or without 1.5 per cent purified agar (purified according to the method of Robbins<sup>17</sup>) was used as such and in various other forms (modified by substituting other sources of nitrogen for the asparagine) in the following vitamin studies.

### *A. Qualitative Studies on Basal Agar with Vitamin Additions*

"Basal Agar A" was prepared by adding 1.5 per cent purified agar to the asparagine broth, "Basal Broth A," previously described. The final pH of the autoclaved medium varied between 6.8 and 7.0 and maintained this range after the various vitamin additions. Filtered vitamin solutions were prepared at the beginning of each experiment, and equal volumes of vitamin solutions, containing vitamins in varying concentrations and combinations, were added to tubes containing 10 cc. of the melted and partially cooled agar, which were then rotated and slanted.

Washed inoculum was prepared from growth in heart infusion broth ("Difco"). It was found necessary to wash the mycelium four to five times with sterile distilled water before all traces of the broth medium were removed. However, even after many washings, a tiny shred of mycelium always carried sufficient nutriment so that a small amount of growth on the control basal agar tubes could be detected. This "fuzzing" of the inoculum was found to be advantageous, as proof was thus afforded that the inoculum was alive. Growth of one millimeter or less was considered negative. All cultures were allowed to grow at room temperature for 10 weeks.

Twenty strains of faviform trichophytons were tested for ability to grow

on the basal agar as well as on basal agar with various vitamin additions. The results indicated that: (1) seventeen of the strains had similar vitamin requirements, namely, inositol and molecular thiamine; (2) one strain, # 18, required pyridoxine in addition to inositol and molecular thiamine; and (3) two strains, # 19 and # 20, showed no essential vitamin requirements, growing equally well on the basal agar with or without vitamin additions. (For descriptions of these strains consult, "List of Strains.")

1. *Seventeen strains which require inositol and thiamine.* This group includes 16 recently isolated strains as well as a stock strain, # 17, *T. discoides* (Papegaay) obtained from the Central Bureau for Fungus Cultures, Holland. These strains showed morphological and cultural characteristics typical of *T. faviforme* of the *album*, *discoides*, and *ochraceum* varieties.

For these strains, inositol has some effect alone, but thiamine is effective only in the presence of inositol. Pyridoxine has no effect alone or when

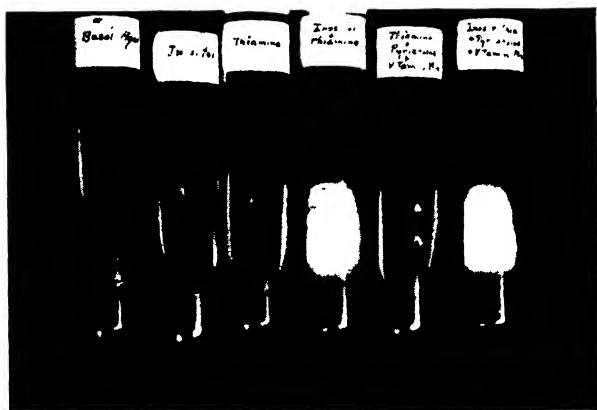


FIGURE 10. *Trichophyton faviforme*, strain # 11, which requires inositol and thiamine for growth. Addition of pyridoxine and "vitamin mixture" does not change the amount of growth.

added to medium containing super-optimal amounts of inositol and thiamine. None of the other vitamins tested, included in "vitamin mixture" (calcium pantothenate, 100 gamma per cc., riboflavin, 100 gamma per cc., nicotinamide, 100 gamma per cc., biotin 0.05 gamma per cc., para-aminobenzoic acid, 100 gamma per cc., choline chloride 100 gamma per cc., and folic acid 100 gamma per cc.), showed any effect when tested singly or in all possible combinations or when added with super-optimal doses of inositol and thiamine (FIGURE 10).

Inositol was not effective for certain strains in amounts less than 10 gamma per cc. The amount of growth with 10 gamma per cc. was very small and for the most part subsurface. It was always definitely more growth than on control tubes, however, and is recorded as one plus. Further increase in the inositol did not increase the amount of growth. As a result of these findings, 100 gamma of inositol (ten times the amount which showed this effect) was used as a super-optimal dose to produce the maximum effect of this vitamin in this medium.

Thiamine showed no effect alone at a concentration of 0.5 gamma per cc. or even when increased to 500 gamma per cc. In the presence of a super-optimal dose of inositol, however, 0.5 gamma of thiamine per cc. had as much effect as a larger dose of this vitamin, producing a four-plus growth of all strains. Five gamma of thiamine per cc. was taken as a super-optimal dose of this vitamin.

2. *Strain #18, Which Requires Inositol, Thiamine, and Pyridoxine.*

Strain #18, *T. discoides* (Mackinnon), was shown to require pyridoxine in addition to inositol and thiamine. This is in accordance with the findings of Robbins, Mackinnon, and Ma,<sup>10</sup> who studied this strain. Inositol showed a very slight effect alone when present in a dosage of 10 gamma per cc. or more, but neither thiamine or pyridoxine showed any effect unless in the presence of both of the other essential vitamins. In the presence of super-optimal doses of inositol and thiamine, 0.5 gamma of pyridoxine

TABLE 1.  
QUALITATIVE GROWTH STUDIES WITH *T. faviforme* ON ASPARAGINE BASAL  
AGAR WITH VITAMIN ADDITIONS

Strains	Thia- mine 5γ/cc.	Pyri- doxine 5γ/cc.	Inositol 100γ/ cc.	Inositol & thia- mine	Inositol, thia- mine, & pyri- doxine	Thia- mine & pyri- doxine + "vitamin mixture"	Inositol, thia- mine, & pyri- doxine + "vitamin mixture"	Basal agar (control)
#1-#17	0	0	1+	4+	4+	0	4+	0
#18	0	0	1+	1+	4+	0	4+	0
#19 and #20	4+	4+	4+	4+	4+	4+	4+	4+

(1) Amount of growth is indicated by 1 + (extends 2 to 5 mm. from inoculum, and is largely subsurface) and 4 + (heavy growth over the larger part of the slant).

(2) "Vitamin mixture": (gamma per cc.), pantothenate 100, riboflavin 100, biotin 0.05, nicotinamide 100, para-aminobenzoic acid 100, choline 100, and folic acid 100.

(3) For descriptions of strains listed, consult, "List of Strains."

produced as much effect as a larger dose of this vitamin, producing a four-plus growth of this strain. Five gamma per cc. was used as a super-optimal dose. None of the vitamins included in the "vitamin mixture" showed any effect when tested alone or in all possible combinations or when added with super-optimal amounts of inositol, thiamine, and pyridoxine (FIGURE 11).

3. *Strains #19 and #20, Which Showed No Essential Vitamin Requirements.* In contrast to all the other strains, #19, *T. album* (Baudet and Stuhmer), and #20, *T. ochraceum* (Boedijn), stock strains obtained from the Central Bureau for Fungus Cultures, Holland, were found to have no essential vitamin deficiencies and grew well on the basal agar. No stimulation was observed following the addition of inositol, thiamine, pyridoxine, or any of the vitamins included in "vitamin mixture."

For all of these strains, the basal agar alone (or basal agar with supra-optimal amounts of required vitamins) was not able to produce growth comparable to that obtained on certain natural mediums. Addition of peptones, heart infusion, Bacto-tryptose, yeast, or liver extracts to the basal



agar not only replaced the essential vitamins, where these were required, but in all cases produced a heavier and more rapid growth. This suggests that there are other important nutritional factors required to produce maximum growth.

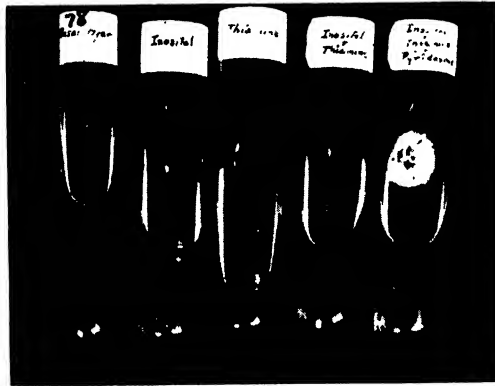


FIGURE 11. *Trichophyton discoides* (Mackinnon) strain #18 which requires pyridoxine in addition to inositol and thiamine.

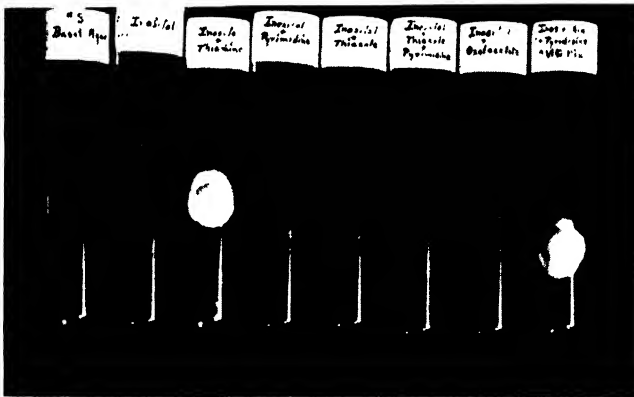


FIGURE 12. *Trichophyton faviforme* strain #8 which requires inositol and thiamine for growth. The thiamine must be present in the molecular form and cannot be replaced by thiazole, pyrimidine, or a combination of these two. Oxalacetate shows no action.

4. *Influence of Different Nitrogen Sources on the Vitamin Requirements.* Ammonium chloride, 0.5 gm. per liter, was substituted for asparagine in the basal agar. (This medium is designated as "Basal Agar B.") Also, a basal agar was prepared in which 0.05 per cent of vitamin-free casein hydrolysate ("Smaco") was used in place of asparagine ("Basal Agar C"). On both of these mediums, the essential vitamin requirements of the strains studied were found to be the same as on "Basal Agar A," which contained asparagine as the nitrogen source. None of the strains except #19 and #20 would grow on these mediums unless the essential vitamins, inositol and thiamine (or inositol, thiamine, and pyridoxine in the case of strain #18), had been added.

Although all of the strains apparently could utilize the inorganic nitrogen present in the ammonium chloride medium, the growth was very scant and did not compare with growth obtained when either asparagine or casein hydrolysate were present.

5. *Use of Component Parts of the Thiamine Molecule.* It has been reported<sup>10</sup> that the Mackinnon strain of *T. discoides* requires intact thiamine and cannot utilize pyrimidine or thiazole separately or in equimolar combination. It was of interest to determine whether the other strains studied in this series were similar in this respect.

Pyrimidine, as 2 methyl 5 ethoxy methyl-6 amino pyrimidine, and thiazole, as 2 amino thiazole, were added singly and in combination, 300 gamma each, to the basal agar tubes with inositol. Growth on all tubes was very meager and comparable to that obtained with inositol alone. Thus, these thiamine requiring strains were shown to need the intact molecule which could not be substituted for by either pyrimidine or thiazole or a combination of the two. The experiment was controlled by including in the protocol two thiamine-requiring fungi, *Phytophthora cinnamomi*, which requires the intact molecule, and *Phycomyces blakesleeana*, which is able to synthesize thiamine from thiazole and pyrimidine. It was also shown that oxalacetate will not substitute for thiamine (FIGURE 12).

#### *B. Characteristics of the Growth on Basal Agar with Added Vitamins.*

As described above, none of the vitamin-deficient strains produced any growth beyond a slight fuzzing of the inoculum on the vitamin-free basal agar with asparagine, casein hydrolysate, or ammonium chloride as nitrogen source. With the addition of inositol, 10 gamma per cc., a small amount of growth was produced in all cases. This was largely submerged and consisted of fine, branching mycelium extending into the medium for a distance of 2 to 5 millimeters.

The addition of thiamine to the basal medium containing inositol greatly altered the character as well as the amount of the growth. The seventeen strains which required inositol and thiamine produced vigorously growing colonies on this medium. The growth was compact and heavy and was generally covered with a thick, white, fluffy, aerial mycelium. The mycelium appeared more regular than on Sabouraud's dextrose agar, and the number of chlamydospores was greatly reduced. The production of reproductive spores varied greatly with the strain. All of the strains showed microconidia and, in some strains, the aerial mycelium was loaded with them (FIGURE 13). On the whole, they were more numerous than on rice grains, on which medium these structures have been described in detail previously. Macroconidia were produced in small numbers by all of the strains on the vitamin-enriched basal agar. They were characteristically very small and delicate. Two strains produced macroconidia in great abundance on the basal agar with inositol and thiamine. These were long and slender, with long tapering ends, a characteristic bean pod-like structure (FIGURE 14).

Strain #18, *T. discoides* (Mackinnon), showed very little growth on the



FIGURE 13. *Trichophyton faviforme*, strain #8, on basal asparagine agar enriched with inositol and thiamine. Note large number of microconidia. 80X

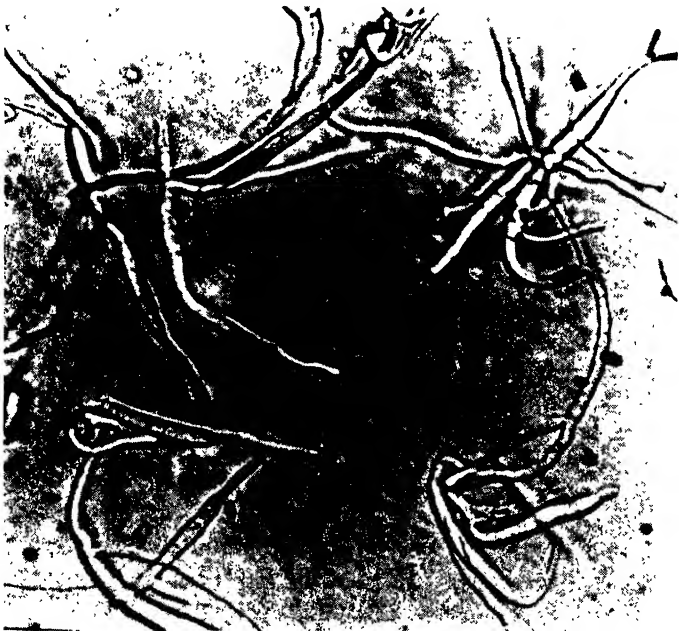


FIGURE 14. *Trichophyton faviforme*, strain #8, on basal asparagine agar enriched with inositol and thiamine. Note large number of macroconidia. 400X

basal agar containing inositol and thiamine, the amount of growth being the same as when inositol was present alone. Addition of pyridoxine, however, to the medium containing supra-optimal doses of inositol and thiamine, stimulated this strain to grow vigorously and to produce colonies which were comparable to those produced by the other strains on basal agar with inositol and thiamine. Both microconidia and a few irregular macroconidia were seen.

Strain # 19, *T. album* (Baudet and Stuhmer), grew on the basal mediums without vitamin additions. Growth on these mediums, however, was almost always completely glabrous. The colonies were raised and heavily folded, frequently being ballooned-out so that the inner areas were hollow. The growth was dry and crumbly and consisted of masses of chlamydospores and fragments of irregular, faviform hyphae. No microconidia or macroconidia were ever seen on this medium. Additions of vitamins—large amounts of thiamine, inositol, and pyridoxine, as well as all the vitamins included in “vitamin mixture”—had no effect in increasing the amount or changing the character of this growth.

Since this strain was autotrophic for the vitamins, in contrast to the heterotrophy exhibited by the strains described above, a study was made of its colonial and microscopic morphology to determine whether it could be set apart on this basis also. It was found to differ in the following ways: (1) it grew at a faster rate, producing a thick colony with many foldings which reached a diameter of 80 millimeters in 6 weeks; (2) it was habitually covered with a downy, white aerial growth even on the simple sugar mediums; (3) the growth was only slightly stimulated by the various enrichments added to the sugar mediums; and (4) it produced microconidia in greater abundance than did any other strain. It seems probable that all of these characteristics may be reflections of the fact that this organism is autotrophic for the vitamins, as the other strains show these same characteristics when grown on basal agar supplied with optimal amounts of the vitamins which they require. The completely glabrous, degenerate type of growth which is produced on basal agar, however, suggests that, although this organism is autotrophic for the vitamins studied, it is lacking in ability to produce some other substances present in natural mediums which are necessary for good growth and the production of spores. Our results indicate that this deficiency cannot be overcome by large amounts of any of the vitamins tested or by casein hydrolysate.

A similar glabrous growth on the basal agar, even in the presence of large quantities of vitamins and casein hydrolysate, is also characteristic of six strains of *T. schoenleini* which we have studied. The close relationship of strain # 19 to *T. schoenleini* will be discussed later.

Strain # 20, *T. ochraceum* (Boedijn), also grew well on the basal agars and was not stimulated by any of the vitamins tested. Some increase in amount of growth was observed on the enriched mediums. On all mediums, this strain produced a completely glabrous, raised cerebriform to highly verrucose colony which at first appeared a light tan but later developed a grey or dark brown pigment, especially in the center of the colony. Micro-

scopically, it consisted of a faviform type mycelium and large numbers of chlamydospores. This strain, obtained from the fungus collection in Holland, has apparently lost the ability to produce the yellow-ochre pigment described by Sabouraud for this species. It has also lost the ability to produce a downy culture with spores on whole grain mediums, described by Lebasque.<sup>3</sup> Although this strain showed no essential vitamin deficiencies, there seems to be no reason to consider it as distinct from two strains of *T. faviforme* (variety *ochraceum*) which we have isolated, which require thiamine and inositol and produce downy cultures with spores on vitamin enriched mediums.

### C. Quantitative Studies in Basal Broth with Vitamin Additions

Using the buffered "Basal Broth A," containing dextrose, magnesium sulfate, and asparagine, several of the strains were studied in order to determine the quantitative effect of addition of the essential vitamins and to compare the maximum growth obtained on vitamin-enriched synthetic medium to that of a natural medium, heart infusion broth ("Difco").

The "Basal Broth A" was prepared, adjusted to pH 7.0, and sterilized by filtration. Fifty cc. amounts were placed in 150 cc. Erlenmeyer flasks, and the freshly prepared vitamin solutions were added in excess (supra-optimal amounts): inositol 100 gamma per cc., thiamine 5 gamma per cc., and pyridoxine 5 gamma per cc. Other vitamins included in "vitamin mixture" were added singly and in all combinations in comparable amounts.

Three of the inositol- and thiamine-deficient strains were studied in this experiment: strains #1 and #5, isolated in Pennsylvania, and #17, *T. discoides* (Papegaay), a stock strain from Holland. On Sabouraud's dextrose agar, strain #1 habitually produced the *album* variety of colony; while strain #5 was of the *discoides* variety. Also studied in this series was strain #18, *T. discoides* (Mackinnon), which required pyridoxine in addition to inositol and thiamine, and strain #19, *T. album* (Baudet and Stuhmer), a stock strain from Holland which had not shown any vitamin deficiencies.

The flasks were inoculated with tiny shreds of washed mycelium, prepared as described above, and the cultures allowed to grow at room temperature, being shaken at intervals in order to keep the growth of a uniform submerged type. After 10 weeks, the entire contents of the flasks were filtered through previously weighed scintered glass filter cups. The retained mycelium was washed with at least 200 cc. of distilled water and packed into a compact mass on the bottom of the cup. The cups were dried in a constant-temperature oven at 110°C. for two hours and, after cooling, reweighed on an analytical balance. The weight of the mycelium was calculated. All glassware was cleaned with acid cleaning solution, rinsed well in distilled water, and sterilized by dry heat. TABLE 2 gives a summary of these tests.

None of the vitamin-deficient strains showed any growth in the basal broth beyond a small amount of fuzzing which indicated that the inoculum was alive. Strain #19 produced considerable growth, approximately 28.23 mg. (average of 6 tests). The single vitamins or the vitamin combinations showed no stimulating effect on this strain, and approximately the same

TABLE 2  
QUANTITATIVE GROWTH STUDIES WITH *T. Furiforme* IN ASPARAGINE BASAL BROTH WITH VITAMIN ADDITIONS

Strain	Thiamine 5γ/cc.	Pyridoxine 5γ/cc.	Inositol 100γ/cc.	Inositol & thiamine	Inositol, thiamine, & pyridoxine	Thiamine & pyridoxine + "vitamin mixture"*	Inositol, thiamine, & pyridoxine + "vitamin mixture"*	Heart infusion broth	Heart in- fusion broth + inositol, thiamine, & pyridoxine	Basal broth (control)
#1 ( <i>album</i> variety) Penna.†	0	0	less than 1 mg.	14.61 mg.	14.39 mg.	0	15.96 mg.	21.49 mg.	39.06 mg.	0
#5 ( <i>discoïdes</i> variety) Penna.†	0	0	1.59 mg.	17.51 mg.	18.34 mg.	0	18.16 mg.	45.02 mg.	91.70 mg.	0
#17 <i>T. discoïdes</i> (Papegaay) Holland†	0	0	1.76 mg.	29.17 mg.	28.96 mg.	0	27.60 mg.	48.65 mg.	85.15 mg.	0
#18 <i>T. discoïdes</i> (Mackinnon) Uruguay†	0	0	1.4 mg.	1.35 mg.	19.70 mg.	0	17.17 mg.	37.15 mg.	56.85 mg.	0
#19 <i>T. album</i> (Baudet- Stuhmer) Holland†	29.18 mg.	27.62 mg.	30.0 mg.	31.19 mg.	30.45 mg.	27.15 mg.	25.20 mg.	72.42 mg.	73.83 mg.	28.23 mg.†

\* "Vitamin mixture": (gamma per cc.), pantothenate 100, riboflavin 100, biotin 0.05, nicotinamide 100, para-aminobenzoic acid 100, choline 100, and folic acid 100.

† Averages of 6 weighings—dried mycelium from 6 flasks.

‡ Averages of 4 weighings—dried mycelium from 4 flasks.

average amount of growth occurred in flasks with and without added vitamins. The increased growth in the heart infusion broth as compared to the growth in the basal asparagine broth may be attributed to the presence of a larger supply of available nitrogen or the presence of unknown growth factors which cannot be identified with any of the vitamins studied.

Growth of the vitamin-deficient strains corresponded well with the results obtained on the basal agar slants in the previous experiment. The three strains studied which had shown growth with inositol and thiamine, #1, #5, and #17, showed even more clearly here that these vitamins were required and that addition of pyridoxine, as well as the other vitamins tested, had no effect on the amount of growth produced. The small amounts of growth produced by inositol alone, never more than 2 mg., corresponded to the small amount of subsurface growth which had been observed on the basal slants containing this vitamin.

Strain #18 was again shown to require pyridoxine in addition to inositol and thiamine. The small amount of growth produced by inositol alone was less than 2 milligrams. None of the other vitamins tested had any effect on the amount of growth produced.

All of the strains showed considerable increase in growth in the flasks containing heart infusion broth. However, even more growth could be obtained with the vitamin-deficient strains when super-optimal amounts of the required vitamins (based on the dosages of these vitamins which would produce maximum growth in the basal asparagine medium) were added to the heart infusion broth. This indicated that the required vitamins were not present in sufficient amounts to produce maximum growth in this medium. As Schopfer and Blumer<sup>18</sup> have shown for *Phycomyces blakesleeanus*, the action of the vitamins is dependent on the medium. By increasing the protein content, the optimal dosage of required vitamins is raised and the production of dry matter is increased.

Titration was made in asparagine basal broth in order to determine the smallest amounts of the essential vitamins which would permit growth of several of the strains studied.

"Basal Broth A" was placed in 10 cc. amounts in test tubes (150 × 18 mm.) and sterilized by autoclaving. The vitamin to be titrated was added aseptically in a volume of 10 cc. to one of these tubes, mixed, and serially diluted through a series of 30 tubes, the amount of vitamin thus being halved by each transfer. Four titrations were carried out for each of the four strains studied and these were done in duplicate: (1) titration of inositol alone; (2) titration of inositol in the presence of thiamine (1 cc. of a standard thiamine solution being added to each tube at the completion of the dilution procedure, giving a final concentration of 5 gamma per cc.); (3) titration of thiamine in the presence of inositol (1 cc. of a standard inositol solution being added to each tube at the completion of the dilution procedure, giving a final dilution of 100 gamma per cc.); and (4) titration of pyridoxine in the presence of thiamine and inositol. Control tubes contained basal broth alone and basal broth with each vitamin singly and in all combinations in supra-optimal amounts. All tubes were brought to equal volume with sterile distilled water.

Strains #1, #5, #18, and #19 were chosen as test organisms. Inoculations were made with minute pieces of washed mycelium and the cultures were allowed to grow at room temperature for 6 weeks. The results are summarized in TABLE 3.

None of the vitamin-deficient strains grew in basal broth control tubes or in tubes containing only thiamine and pyridoxine. Some growth occurred in tubes containing inositol alone. The end point of growth was clear cut in all cases and occurred for strain #1 in a dilution containing 9.75 gamma per cc., for strain #5 at 1.22 gamma per cc., and for strain #18 at 4.8 gamma per cc. Growth in tubes containing inositol was represented by small balls of fluffy growth in the bottoms of the tubes—never more than 2 milligram dry weight. Increasing the amount of inositol did not produce a corresponding increase in the amount of growth.

TABLE 3.

SMALLEST AMOUNTS OF THE ESSENTIAL VITAMINS WHICH WILL ALLOW THE GROWTH OF *T. faviforme* IN ASPARAGINE BASAL BROTH

Strain	Inositol	Thiamine (in the presence of excess inositol—100 $\gamma$ /cc.)	Pyridoxine (in the presence of excess inositol and thiamine—5 $\gamma$ /cc.)
#1	gamma per cc. 9.75	gamma per cc. 0.002	gamma per cc. no effect of pyridoxine observed
#5	1.22	0.004	no effect of pyridoxine observed
#18	4.8	no effect of thiamine observed	0.002
#19	no effect of inositol observed	no effect of thiamine observed	no effect of pyridoxine observed

For strains #1 and #5, the presence of super-optimal amounts of thiamine greatly increased this growth, and the same dilutions of inositol which had shown only a small, fluffy ball at the bottoms of the tubes were filled with mycelium. Growth fell off sharply, however, at the same dilutions of inositol as it had when inositol had been present alone. The action of inositol seems to be concerned with the initiation of growth and development of the mycelium to a certain point only. In the presence of thiamine, the same minimal amounts of inositol are required to initiate the growth, but the development of the mycelium continues with greater utilization of the medium.

No growth of any of the strains was obtained with thiamine alone, even in dilutions containing 625 gamma per cc. In the presence of supra-optimal doses of inositol, however, heavy growth of strains #1 and #5 occurred in all tubes containing 0.02 gamma or more thiamine per cc. In succeeding dilutions, which contained less and less thiamine, the growth could be seen



to decrease gradually in amount, the end point of the effect of this vitamin occurring in dilutions containing 0.002 and 0.004 gamma of thiamine per cc. Beyond these dilutions, only a small amount of growth occurred in all tubes which could be accounted for by the inositol present, which, as shown above, has some action on these strains without thiamine. Thus, thiamine alone showed no action on the growth of these strains. But, in the presence of inositol, the amount of growth was roughly proportional to the amount of thiamine present until a maximum amount of growth for this medium was obtained.

Titration of pyridoxine in the presence of inositol and thiamine did not give any evidence of stimulation by this vitamin except for strain #18, which has been shown to require pyridoxine.<sup>10</sup> With this strain, heavy growth occurred in all tubes containing 0.01 gamma or more of pyridoxine per cc. In succeeding dilutions, which contained less and less pyridoxine, the growth could be seen to decrease gradually in amount, the end point of the effect of this vitamin occurring in a dilution containing 0.002 gamma of pyridoxine per cc. The very small amounts of growth which occurred in all tubes beyond this dilution could be accounted for by the inositol present, which has some effect by itself.

Strain #19, which had not been shown to be vitamin-deficient by previous methods, grew equally well in all tubes, thus showing no stimulation by any of the vitamins tested. This strain was also tested using dilutions of biotin in the "Basal Broth A" and also in "Basal Broth B" in order to determine whether biotin had any effect as reported by Schopfer and Blumer<sup>12</sup> for a strain of *T. album*. No effect of biotin, either in asparagine basal broth or in ammonium chloride basal broth, was observed.

### *Part III. Growth Characteristics of Other Species in the Faviform Trichophyton Group*

A brief survey was made of other species in the faviform trichophyton group in an attempt to relate these forms on the basis of their cultural characteristics and nutritional requirements. The following strains were studied: (1) six strains of *T. schoenleini* recently isolated from cases of favus, and (2) several cultures obtained from the Central Bureau for Fungus Cultures, Holland: *T. immergens* (Milochevitch), *Favotrichophyton decipiens* (Boedijn and Verbunt), *T. bullosum* (Lebasque), and *T. equinum* (Gedolst).

#### *A. Trichophyton schoenleini*

The six strains of *T. schoenleini* studied showed cultural characteristics typical of those described for this species. On Sabouraud's dextrose and maltose agar, these strains produce slow-growing, heaped, and folded colonies which are at first largely glabrous, but later usually develop some white powder and in some areas a short white down. Irregular, branching mycelia grow in the depths of the medium and form ragged edges about the raised colonies. The colony type is variable, a single strain showing reversible changes from completely glabrous to downy colonies re-

sembling, in this respect, the colonial variations shown for single spore cultures of *T. faviforme* (var. *album*, *discoides*, and *ochraceum*). Bacterial contamination of the medium, particularly with the staphylococcus, stimulates the growth of *T. schoenleini* so that more white powder and down are obtained. Growth on mediums enriched with heart infusion, Bacto-tryptose, yeast, or liver extracts caused some increase in the amount of growth, the colonies being heavier and more folded and often showing a raised, regular border. Microscopically, the growth on both the Sabouraud's dextrose agar and the enriched mediums was composed of thick, irregular, highly branched mycelium ("chandeliers") and many chlamydospores. Microconidia were extremely rare, except on rice grains, where they were found in small numbers. They were small and pyriform and appeared similar to those seen in other trichophyton species. Macroconidia were not observed on any medium.

All six strains of *T. schoenleini* were able to grow on basal agars with ammonium chloride, asparagine, or casein hydrolysate as nitrogen source. On these basal agars, they produced completely glabrous, much folded, ballooned-out colonies which became tan to dark brown with age and were dry and crumbly. They consisted of irregular fragments of twisted hyphae and many highly irregular chlamydospores. The amount of growth on the basal mediums varied considerably with the strain, two of which grew rather feebly on these mediums. Addition of large amounts of the vitamins studied had no effect in changing the character or increasing the amount of the growth of any of these strains. Although these strains of *T. schoenleini* appear to be completely autotrophic for the vitamins studied, the completely glabrous, degenerate, and in some instances very meager growth which several of the strains produce on the basal agars suggests that they are lacking in ability to produce some other substances present in natural mediums which are necessary for good growth and spore production (FIGURE 15).

In all respects, except for their slower and usually more glabrous growth and poor ability to produce microconidia, these *T. schoenleini* strains resembled quite closely strain #19, *T. album* (Baudet and Stuhmer), previously described.

### *B. Trichophyton immergens*

*T. immergens* is described by Milochevitch<sup>19</sup> as follows: "On Sabouraud's glucose medium it produces an irregular greyish colony with elevated center and is covered with a short, scant, white down. Around the center there is a large zone of immersing rays, large and uneven. The reverse side of the colony is a bright, clear yellow." On maltose agar a fluffy white aerial growth is described. The aerial growth consists of sterile mycelium and the submerged growth shows a faviform hyphae and many chlamydospores. By growing the cultures on kernels of corn, Milochevitch obtained microconidia, *en thyse* and *en grappe*, but no macroconidia. The lesions produced are usually of a deep suppurative type, but typical favic scutulae have been described in several cases of infection due to this organism.

The strain of *T. immergens* obtained from the stock collection in Holland produced colonies similar to those described by Milochevitch. No stimulation of growth was observed on enriched mediums and no microconidia or macroconidia were obtained, the strain appearing to have become completely pleomorphic. The growth on the basal agars was similar in all respects



FIGURE 15. *Trichophyton schoenleinii*: tube #10—growth on basal asparagine agar; tube #14—growth on basal asparagine agar plus inositol, thiamine, and pyridoxine; tube #16—growth on basal asparagine agar plus "vitamin mixture;" tube #19—growth on basal asparagine agar plus inositol, thiamine, pyridoxine, and "vitamin mixture."



FIGURE 16. *Trichophyton equinum* (Boedijn) requires nicotinic acid for growth. This can be substituted for by nicotinic amide or 1-tryptophane.

to the growth on both the simple sugar mediums as well as on the enriched mediums. Thus, no deficiencies could be detected in this strain.

### C. *Favotrichophyton decipiens*

*Favotrichophyton decipiens* (Boedijn and Verbunt), also obtained from the fungus collection in Holland, produced a flat, greyish colony with a small raised center and many fine radial grooves. The colony was covered with a scant but long greyish decumbent aerial mycelium. Microscopically

it consisted of a faviform mycelium and many chlamydospores. No stimulation of growth was observed on enriched mediums and no microconidia or macroconidia were obtained—this strain apparently had also become pleomorphic. The growth on the basal agars was similar in all respects to the growth on both the simple sugar mediums as well as on the enriched mediums. Thus, this strain also showed no apparent deficiencies.

#### *D. Trichophyton bulbosum*

*T. bulbosum* was described by Lebasque<sup>3</sup> as a very slow-growing, glabrous culture with a highly irregular surface tending to show dome-shaped protrusions. Microscopically, it consisted of faviform hyphae and many chlamydospores. Lebasque obtained downy cultures on the natural mediums and both microconidia and macroconidia were described.

The strain of *T. bulbosum* obtained from the fungus collection in Holland produced cultures resembling those described by Lebasque. It never formed a downy culture, however, and no microconidia or macroconidia were obtained on enriched or whole grain mediums. This strain would not grow on any of the basal agars and was not stimulated by large doses of any of the vitamins used in these studies. Further study is being made to determine the essential growth requirements of this organism.

#### *E. Trichophyton equinum*

*T. equinum* (Geddoelst) is described by Lebasque<sup>3</sup> as a large-spored ectothrix organism obtained from cattle or horses which produces a downy to fluffy culture on the usual laboratory mediums. The fluffy growth, as in the case of *T. immersens*, consists of sterile hyphae. By growing the culture on whole grains, Lebasque obtained large numbers of microconidia and macroconidia in the 12 strains which he studied.

The strain of *T. equinum* obtained from the fungus collection in Holland showed a white, fluffy growth of sterile mycelium on the simple sugar mediums, as well as on the enriched mediums, and produced a bright yellow pigment on the reverse side of the colony. Only a few microconidia and an occasional macroconidium were observed on rice grains. The organism would not grow on "Basal Agars A and B," which contained asparagine and ammonium chloride. However, a very small amount of submerged growth was obtained on the "Basal Agar C," which contained casein hydrolysate. By adding various vitamin solutions to the basal mediums, it was found that good growth occurred when either nicotinic acid or nicotinamide was added. Growth on the nicotinic acid-enriched basal agars was white and fluffy and composed of sterile hyphae, thus resembling the growth of this organism on other mediums. No stimulation of spore production or further stimulation of growth was obtained by adding large doses of inositol, thiamine, pyridoxine, or any of the vitamins included in "vitamin mixture," to the basal agars enriched with nicotinic acid. Also, none of these substances could substitute for nicotinic acid or nicotinamide.

The fact that a small amount of growth occurred on "Basal Agar C" suggested that either the casein hydrolysate was contaminated with small

amounts of nicotinic acid or one of the amino acids present in casein might have stimulating properties for this organism. It was found that l-tryptophane had this property, and that, when added to "Basal Agars A or B," good growth of *T. equinum* could be obtained even in the absence of nicotinic acid or nicotinamide (FIGURE 16). d-Tryptophane was found to be inactive. The type of growth obtained with l-tryptophane was similar in all respects to that obtained when nicotinic acid was present. The combination of nicotinic acid and tryptophane did not change the character of this growth.

Titration was made in ammonium chloride basal broth to determine the smallest amount of nicotinic acid, nicotinamide, or l-tryptophane which would permit the growth of the strain of *T. equinum* studied. Results of the titrations were as follows: (1) both nicotinic acid and nicotinamide were active to a dilution containing 0.05 gamma per cc. and no growth occurred in higher dilutions, and (2) l-tryptophane was active in a dilution containing 125 gamma per cc. and no growth occurred in higher dilutions.

This substitution of an amino acid for nicotinic acid suggests the possibility of tryptophane as a precursor for this vitamin growth factor. Krehl *et al.*,<sup>20</sup> in their studies of the pellagragenic effect of corn, were the first to show a possible interchangeable role of these two substances. Later experiments by Rosen *et al.*<sup>21</sup> and those of Sarett and Goldsmith<sup>22</sup> indicated that tryptophane may be an important precursor of nicotinic acid in rats as well as in humans and may explain the antipellagragenic activity of certain foods such as milk, which is low in nicotinic acid but rich in protein. Bonner and Beadle<sup>23</sup> have recently described three X-ray mutants of neurospora which require nicotinic acid for growth. On the basis of their studies, Woolley<sup>24</sup> has postulated that, if tryptophane is a precursor of nicotinic acid in neurospora, it must be three enzymatic steps removed from the vitamin.

The possibility that tryptophane may be the essential factor required by *T. equinum*, with nicotinic acid functioning in some enzyme system necessary for the production of this amino acid, is also to be considered.

According to Schopfer,<sup>25</sup> no naturally occurring fungi have been reported to require nicotinic acid. Further strains must be obtained and studied before it can be determined whether this requirement reported here for a strain of *T. equinum* is characteristic for this species.

#### Summary and Conclusions

(1) As a result of morphological and cultural studies employing single-spore strains of recently isolated faviform trichophytons, as well as a study of the growth requirements of these strains, it seems apparent that *T. album*, *discoides*, and *ochraceum* are variants of a single species. We propose that they be classified as *T. faviforme* (varieties: *album*, *discoides*, and *ochraceum*).

(2) On the usual sugar mediums, growth was very slow and meagre and of the glabrous, nonsporulating type characteristic of the faviform group. However, cultures on enriched and whole grain mediums demonstrated

that these strains could produce vigorously growing, downy to fluffy colonies with a regular mycelium and microconidia and macroconidia characteristic of the *Trichophyton* group.

(3) Synthetic, vitamin-free mediums were used as a basis for studies of the vitamin requirements of the recently isolated as well as stock strains of *T. faviforme* (var. *album*, *discoides*, and *ochraceum*). A group of closely related species were included in these studies. One may postulate that a gradual loss of synthetic abilities has occurred in this group of organisms as suggested by the following findings:

(a) Autotrophic strains such as *T. immergens* (Milochevitch) and *Favotrichophyton decipiens* (Boedijn and Verbunt), two members of the group which produce slightly downy cultures on the usual mediums, grow as well on chemically defined, vitamin-free mediums as on natural mediums. However, these strains require certain substances present in natural products in order to produce spores. This deficiency cannot be overcome by large amounts of any of the vitamins studied or by casein hydrolysate.

(b) Autotrophic strains such as six strains of *T. schoenleini* studied and two stock strains, *T. album* (Baudet and Stuhmer) and *T. ochraceum* (Boedijn), although able to grow to some extent on synthetic, vitamin-free mediums, produce completely glabrous, degenerate colonies on these mediums. On certain natural mediums, they produce a more luxuriant, regular growth with aerial mycelium and spores. Here again, the deficiency cannot be overcome by large amounts of any of the vitamins studied or by casein hydrolysate.

(c) A partially heterotrophic strain classified as *T. album* has been shown by Schopfer and Blumer<sup>12</sup> to have the ability to grow very slightly in a vitamin-free medium. They found, however, that its growth could be greatly stimulated by a combination of biotin, inositol, thiamine, and pyridoxine. They were able to substitute a combination of pyrimidine and thiazole for the thiamine growth factor.

(d) A heterotrophic strain, *T. equinum* (Geddoelst), was found to require one vitamin, nicotinic acid or the closely related compound nicotinamide, in order to grow in a synthetic vitamin-free medium with either ammonium chloride or asparagine as nitrogen source. The vitamin requirement, however, can be dispensed with in the presence of l-tryptophane. This substitution of the amino acid for nicotinic acid or vice versa, suggests either that tryptophane may be a precursor of nicotinic acid in the metabolism of this fungus, or that nicotinic acid may function in some enzyme system necessary for the production of tryptophane. Further strains must be obtained and studied before it can be determined whether this requirement is characteristic of the species, *T. equinum*.

(e) Heterotrophic strains such as the sixteen recently isolated cultures of *T. faviforme* of the *album*, *discoides*, and *ochraceum* varieties as well as a stock strain, *T. discoides* (Papegaay), were shown to require two vitamins, inositol and thiamine, in order to grow in synthetic, vitamin-free mediums with ammonium chloride, asparagine, or casein hydrolysate as nitrogen source. The thiamine could not be substituted for by pyrimidine or thiazole

or a combination of these two component parts of the thiamine molecule. Inositol produced some effect alone, and seemed to be concerned with the initiation of growth and development of the mycelium only to a certain point. Thiamine showed no action alone, but, in the presence of an adequate supply of inositol, it stimulated these strains to produce rapidly growing colonies covered with a thick white aerial mycelium which contained microconidia and macroconidia typical of the trichophyton group.

(f) A heterotrophic strain, *T. discoides* (Mackinnon), has been shown by Robbins, Mackinnon, and Ma<sup>10</sup> to require three vitamins in order to grow on synthetic, vitamin-free medium with asparagine as nitrogen source. These are inositol, thiamine, and pyridoxine. The thiamine could not be substituted for by pyrimidine or thiazole or a combination of these two component parts of the thiamine molecule. These authors also showed that certain other substances present in natural mediums have a stimulating effect on this strain.

(g) A heterotrophic strain, *T. bulbosum* (Lebasque), would not grow on any of the synthetic vitamin-free mediums and was not stimulated by the addition of large amounts of any of the vitamins studied or casein hydrolysate. The growth requirements of this strain are undetermined.

(4) Quantitative studies with the vitamin-deficient strains indicated that the amount of growth in the asparagine broth containing super-optimal amounts of the required vitamins did not in any case attain the value (dry weight of mycelium) obtained in a natural medium, heart infusion broth ("Difco"). It was further shown that the optimum requirements for the vitamins was dependent on the medium employed.

The smallest amount of inositol which would allow growth in asparagine broth ranged between 1 and 10 gamma per cc. depending on the strain studied which required this vitamin. The amount of growth produced was never more than 2 mg. and could not be increased by further addition of inositol.

The smallest amount of thiamine, in the presence of super-optimal amounts of inositol, which would allow growth in asparagine broth ranged between 0.002 and 0.004 gamma per cc., depending on the strain which required this vitamin. As the thiamine was increased, a corresponding increase in amount of growth was observed. Maximum growth was obtained with 0.02 gamma of thiamine per cc.

The smallest amount of pyridoxine, in the presence of super-optimal amounts of inositol and thiamine, which allowed growth in asparagine broth of the strain which required this vitamin was 0.002 gamma per cc. As the pyridoxine was increased, a corresponding increase in the amount of growth was observed. Maximum growth was obtained with 0.01 gamma of pyridoxine per cc.

The smallest amount of either nicotinic acid or nicotinamide which would allow the growth of *T. equinum* in an ammonium chloride broth was 0.05 gamma per cc. The smallest amount of l-tryptophane which would allow the growth of this organism in the absence of the vitamin growth factor was 125 gamma per cc.

(5) The close relationship of the faviform trichophytons to *T. schoenleini* has been shown, from the early morphological studies by Sabouraud and Bodin to the present study of growth factors. However, clarity in the literature and a better understanding of these organisms and the diseases which they produce may best be accomplished by distinguishing the faviform trichophytons as a group separate from *T. schoenleini*. This is proposed on the following basis:

(a) The faviform trichophytons are, primarily, infectious agents which cause ringworm in cattle and other domestic animals, while *T. schoenleini* causes a specific disease, favus, which is usually transmitted from man to man.

(b) The faviform trichophytons (with few exceptions) produce a deep, suppurative type of lesion in man. The parasitism of the hair is typically large-spored and ectothrix and the hairs are broken off close to their roots. This is in contrast to the usual scutular lesions produced by *T. schoenleini* and the endothrix invasion of the length of the hairs.

(c) The faviform trichophytons are definitely stimulated by enriched or whole grain mediums with production of vigorous growth with microconidia and macroconidia. *T. schoenleini* strains show only slight stimulation on such mediums, and, when this occurs, we have observed only few microconidia and no macroconidia.

(d) Of twenty strains of faviform trichophytons studied, eighteen showed essential vitamin deficiencies for inositol and thiamine and in one instance for pyridoxine as well. One member of the faviform group was shown to require nicotinic acid. None of the six strains of *T. schoenleini* studied showed an essential vitamin deficiency, nor were any of the strains stimulated by large amounts of any of the vitamins studied.

(e) One strain which we have studied, *T. album* (Baudet and Stuhmer), appears to be very closely related to *T. schoenleini* and possibly represents an intermediate stage between the vitamin-deficient faviform trichophytons and the *T. schoenleini* strains. This strain showed no vitamin deficiencies, but it lacked the ability to produce some other substances present in natural products which were required for vigorous growth and production of spores.

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### List of Strains

#### I. *Trichophyton faviforme* (var. *album*, *discoides*, and *ochraceum*).

Strains #1-#17, which require inositol and thiamine:

(a) #1-#8 Isolated from suppurative ringworm in farmers, Pennsylvania. Contact with infected cattle established in all cases. Colonies of three varieties: *album*; *discoides*, and *ochraceum*.

(b) #9 Isolated by Dr. I. Seligman, Baltimore, Maryland. Patient presented a deep, suppurative lesion. Contact with infected animals not known. Colony of the *album* variety.

(c) #10-#15 Isolated by Dr. R. G. Carney, Iowa City, Iowa. Patients presented different types of inflammatory lesions. Contact with infected cattle established in all but one case. Colonies of the *album* and *discoides* varieties.

(d) #16 Isolated by Dr. S. Auchhiesiger, Quito, Ecuador. Cattle in this region are heavily infected with ringworm. Colony of the *album* variety.

(e) #17 *T. discoides* (Papegaay). Stock strain, Central Bureau for Fungus Cultures, Holland.

Strain #18, which requires inositol, thiamine, and pyridoxine:

*T. discoides* (Mackinnon).

Isolated by Dr. J. E. Mackinnon, Montevideo, Uruguay.

Strains # 19 and # 20, which showed no essential vitamin requirements:

*T. album* (Baudet and Stuhmer).

Stock strain, Central Bureau for Fungus Cultures, Holland.

*T. ochraceum* (Boedijn).

Stock strain, Central Bureau for Fungus Cultures, Holland.

II. *Related Species*

(a) Six strains of *T. schoenleini* recently isolated from favus cases by Miss M. E. Hopper, New York Hospital, N.Y.

(b) The following stock strains from the Central Bureau for Fungus Cultures, Holland:

*T. immergens* (Milochevitch)—Milochevitch.

*Favotrichophyton decipiens* (Boedijn and Verbunt)—Boedijn.

*T. bullosum* (Lebasque)—L. P.

*T. equinum* (Gedoeft)—Baudet.

# NEW INSIGHT GAINED IN GENERAL PATHOLOGY AND PRACTICAL MEDICINE BY THE STUDY OF SPOROTRICHOSSES

By Henri Gougerot\*

*Professor of Dermatology and Syphilology, Medical Faculty of the University of  
Paris, France*

The first two cases of sporotrichosis observed were that of Schenck (the fungus of which was classified by Smith in 1898 as *Sporothrix schencki*), followed by that of Hoekten and Perkins in 1900. By clinical analogy, these latter authors connected their case with a clinical observation of Brayton which was not examined bacteriologically. These three American cases had the form of an ascending, gummatous lymphangitis, and iodide therapy was apparently much less effective than in our European cases.

These American observations were unknown in Europe, despite the fact that, in 1901, Foulerton presented an excellent summary of them before the Pathological Society of London. De Beurmann and the author were the first to take up this work in France, in 1906, and make it known there. The discovery of *Sporotrichum beurmanni* rescued the sporotrichoses from oblivion, and, thanks to this research, new cases in man and in horses were published in the United States after 1909 by Burlew, by Trimble and Shaw, by Page, Frothingham, and Paige, by Mohler, by Mervins-Hyde and Davis, by Duque (in Cuba), and by many others.

The first case in France observed by de Beurmann and Ramond (1903), which was caused by an organism called *Sporotrichum beurmanni* by Matruchot and Ramond in 1905, had been forgotten when, with de Beurmann, I had the good fortune to make new observations which formed the basis of my own later work. Dor, of Lyon, was the first to use the term, sporotrichosis, in 1906. However, I proved later that his *Sporotrichum dori* was a *Nocardia*, and the mycologists confirmed this view. Consequently, up to the series of studies by de Beurmann and the author, three proven observations were counted, namely, that of Schenck, of Hoekten and Perkins, and that of de Beurmann and Ramond. These isolated researches had not attracted any attention or following and remained unknown. It was not until the publication of the ten reports by de Beurmann and the author (between 1906 and 1911) that the problem of sporotrichosis was opened to serious discussion and its importance in general pathology and practical medicine pointed out.

It is not the purpose, here, to repeat a review of the field, which has been published before,<sup>1</sup> but to evaluate as completely as possible the new insight gained by the study of sporotrichosis in the above mentioned fields.

## *Parasitology: General Mycology*

The genus *Sporotrichum* has brought light to many questions in general mycology, though it has failed to solve some fundamental problems. Are

\* Translated from the French original by Lothar Salin, former Associate Editor of The New York Academy of Sciences.

the *Sporotricha* degenerated forms of the *Ascomycetes* which have, during their parasitic life with man and the lower animals, lost their higher form of reproduction? Asci have been reported in cultures of *Sporotrichum crateriforme*, but it is very uncertain whether this fungus is a *Sporotrichum*. Sartory took it for an *Endomyces*, and in all the thousands of different *Sporotrichum* cultures which I have handled, I have failed to find any asci, despite all efforts. For this reason, we shall not discuss whether these organisms are degenerated fungi or whether they belong to the lower fungi and have never had a higher form of reproduction.

The classification of the genus *Sporotrichum* is still disputed. Vuillemin,<sup>2</sup> of Nancy, put some in the class *Conidiospora*, in the order *Sporotricha*, with two principal genera, *Sporotrichum* and *Rhinocladium*. He calls *Rhinocladium* the *Sporotriches* of both Schenck and de Beurmann. This change of name was, however, not recognized by Matruchot, who maintains the classification in the genus *Sporotrichum*.<sup>3</sup>

To begin with, Matruchot remarks, "The differences mentioned, such as coloring of the filaments and spores, the mode of attachment of the spores (pedicellate or sessile), are not so remarkable as the diagnoses given in the books suggest. These divers characteristics are so little specialized that they can all be found on the same parasite. The facts presented to us by nature cannot be put into compartments so easily."

From Saccardo, who created the genus *Rhinocladium*, Matruchot takes the definition of the genus *Sporotrichum* ("conidia in ramorum denticulorum apicibus aerogena") as well as examples of several species, namely, *Sporotrichum mycophilum*, *membranaceum*, and *flexuosum*, for which Saccardo specifically notes pedicellate spores. Matruchot also cites other examples taken from his own work, such as *Ctenomyces serratus*, etc. Thus, he thinks that the presence of pedicellate spores, which, according to Vuillemin, would be a characteristic of *Rhinocladium* and distinguish it from *Sporotrichum*, can apply to *Sporotrichum* as well and, consequently, is of no differentiating value.

In support of his opinion, Matruchot supplies a second series of proofs.<sup>3</sup> He shows that *Sporothrix beurmanni* cannot be classified as a *Rhinocladium*, which genus is defined as follows by Costantin: "Fertile blackish filaments ramified in dichotomy, more or less irregular, erect, directed towards the extreme end of the last ramifications of the lateral and terminal spores, which are placed on toothlike projections. The spores are globular or oval-shaped and blackish." However, says Matruchot, in *Sporotrichum beurmanni*, "the fertile filaments are not blackish; they are not branched dichotomously when mature, the spores are not placed exclusively near the extreme end of the terminal filaments, but, rather, on the entire wall of the fertilized branches up to a very great distance from the extreme end. Finally, though the spores may be placed on toothlike projections, they are just as frequently entirely sessile and the filaments whose spores have fallen off bear no trace of how they were originated." The diagnosis of *Rhinocladium*, thus, cannot apply to our parasite, which has to retain the name of *Sporotrichum*—"the natural affinities of *Sporotrichum beurmanni* are elsewhere than in the genus *Rhinocladium*."

We cannot but compliment Professor Matruchot on his argument. Should we, however, distinguish with him between *Sporotrichum schencki* and *beurmanni* (*loc. cit.*), or should they be considered as one species, as was done by Verdin and Vuillemin? The last-named author believes that the cultures of *schencki* which were sent to us in Paris and which we studied for a long time, are actually degenerate and pleomorphic, hence their apparent difference as compared to the *beurmanni*. However, at the end of his discussion, Vuillemin gives the name of *Rhinocladium beurmanni* to the parasites of both Schenck and de Beurmann, stating that "usage has sanctioned the name of *Rhinocladium beurmanni*." Other authors, nevertheless, in making the synthesis, prefer the double name *Sporotrichum schencki-beurmanni*, and, in 1910, I proposed the following table:

<i>Sporotrichum</i> (ancestral)	{	1ST GROUP	{	<i>Sporotrichum schencki</i>	(I)
		<i>Sporotrichum schencki-beurmanni</i> common source of:		<i>Sporotrichum beurmanni</i>	(II)
				and its varieties:	
				<i>Sporotrichum beurmanni</i>	
				var. <i>asteroides</i>	(III)
				<i>Sporotrichum beurmanni</i>	
				var. <i>indicum</i>	(IV)
				<i>Sporotrichum jeanselmi</i>	(V)
		2ND GROUP			
		<i>Sporotrichum gougeroti</i> etc.			

Vuillemin, Grigorakis, and also Dodge<sup>4</sup> made an attempt to change the name of *Sporotrichum gougeroti*, which was established by Matruchot, to *Dematium gougeroti*. Saccardo considers this organism related to *Torula*. Vuillemin's argument was that "the cultures of *Sp. gougeroti* do not contain any conidia" (which is inexact) and "the filaments, generally dull and opaque in appearance, produce budding, colorless globules of variable dimensions." Together with Matruchot and in collaboration with Duché, I have objected to this change of name for two principal reasons, namely: (1) the parasite in question has colored spores, or conidia; (2) the genus *Dematium* is badly defined since "all the Dematiaceae can be incorporated in it as long as only the yeast forms are considered."

Dodge also refused to accept the classification of several *Sporotricha*. Thus, he makes both *Sporotrichum greconis*, described by Mackinnon, and *Sporotrichum fiococci* special varieties of *Sporotrichum schencki*. From *Sporotrichum schencki-beurmanni*, he separates *Sporotrichum cracoviense*, *grigsbyi*, and *coucilmanni*. He eliminates *Sporotrichum equi*, which he considers similar to *Zymonema farciminosum*. He regards as doubtful *Sporotrichum congolense* (Baerts), *Rhinocladium gudyauquilense* (Valenzuela), *Sporotrichum indicum* (Castellani and Chalmers), *Sporotrichum lesnei* (Vuillemin), *Sporotrichum bronchiale* (Montagne), and *Rhinocladium parvulum* (Redaelli).

Guégen asks whether it would not be worth while to create a new genus, *Sporotrichopsis*. To quote from his argument, "The genus *Sporotrichum* is so badly defined, or rather so little defined, that a radical measure seems indicated. This would consist of treating the genus *Sporotrichum* as an abandoned cemetery where no one is buried any longer. By making *Sporo-*

*trichum beurmanni* the type of a new genus, *Sporotrichopsis* (which would be perfectly defined), one would have a solid basis—a center around which other species could be grouped as well as, no doubt, older *Sporotricha*, after somebody has taken the trouble to study their structure.” The presence of “sympodially born conidia,” which has not been noted in the genus *Sporotrichum*, constitutes, according to Guégen, another argument in favor of the creation of a new genus.

The forms in the tissues are different from those in the *in vitro* cultures. The writer believes that he was the first to describe these short forms and to have shown that they are not spores, as other authors thought. These forms are degenerated, due to adaptation in the struggle for existence in the tissues. The writer has also drawn attention to the budding yeast form, similar to that encountered in the blastomycoses.\*

A thorough study of the yeast forms of the sporotrichoses has been made by Charlotte C. Campbell, who showed that this phase persists in Francis's solution (glucose agar with blood, with cystine added), as long as this is incubated at 37°C. On the other hand, transplanted on Sabouraud's agar, a yeast-form *Sporotrichum* will exhibit the typical filamentous form within 48 hours at room temperature.

Splendoré, of São Paulo, has described star-like forms in *Sporotrichum asteroides*, which the author, with Dodge, believes to be a simple variety of *schencki-beurmanni*. Spillman and Gruger, in animals and Greco, in man, have noted radiating actinomycosiform specimens, whose presence in the tissues was also pointed out by Moore and Ackerman.<sup>5</sup> Widal and Abrami discovered the seroagglutinations, complement fixations, and group reactions. This method is useful not only to diagnose sporotrichosis, but it can also, owing to the shortness of the reactions, be used to diagnose other mycoses, particularly actinomycoses. However, I have shown several causes of error here, and likewise in the intradermal reactions with sporotrichin (killed fungus antigen). For example, a simple saprophyte of *Sporotrichum*, in the bucco-pharynx, or even that of a different fungus, perhaps a yeast form in the digestive tube, can, by group reaction, give seroagglutinations of up to 1/150, with medium complement fixations. This would give the erroneous impression that a lesion was of sporotrichotic nature, when actually it is due to an entirely different agent.

The observations of Widal and Weill, and afterwards my own, have shown, through blood cultures, that *Sporotricha* are present in the circulating blood. Blanchetière and the author (1909) made a thorough study of the toxins of the *Sporotricha*, namely, soluble toxins, insoluble toxins (endotoxins), etherosporotrichosine, chlorosporotrichosine, *etc.* We also analyzed their fermentative power on various substances, comparing the different species and stressing their instability in this respect. While these criteria are use-

\* Since 1909, I have requested a revision of this group of the blastomycoses, stressing the fact that the yeast form is common to a variety of very different parasites in their struggle for existence in the tissues. The exascoses, endomycoses, and parendomycoses (thrush), saccharomycoses (mycosis of Busse-Buschke) and parasaccharomycoses, as well as the zymonematoses (Gilchrist's disease), have been treated in collaboration with de Beurmann. For a revision of the old group of blastomycoses, see Soc. Méd. Hôp. 26: 222 and 27: 250 (1909), as well as Trib. Méd., August 7 and 11, 1909. See, also, The Question of Blastomycoses: A Complete Revision of the Old Group. Rev. Derm. Argentina, Special Number in Honor of Dr. B. Sommer: 75. 1916.

ful in bacteriology, they have thus become of rather questionable value in mycology.

De Beurmann, Ravaut, Verdin, and the author have studied sensitizations and group sensitizations, above all the intradermal reactions, and have opened the chapter of polymycoses. This research has led us to realize that the sporotrichoses may take on an appearance similar to that of bacterial infections.

### *Practical Medicine*

From the point of view of practical diagnosis, I have popularized Sabouraud's technique of culture at room temperature on French proof agar, showing that incubated cultures give slower and less constant results (and, above all, less characteristic colonies). Thanks to this technique, the mycological diagnosis of sporotrichoses has become simple and rapid, and has come within the reach of even the most isolated practitioner, since this method of culturing makes it possible to dispense with an incubator, and since, moreover, the macroscopical aspect of the colonies is sufficiently characteristic without necessitating laboratory examination.

In order to speed up bacteriological diagnosis, I have drawn attention to "the run of pus on dry glass." The originating colonies are easy to discover through the thickness of the glass, without any preparation or stain. Also, in order to facilitate the study and identification of the fungi, I have proposed the so-called technique of "dry slides." Finally, in 1908, I showed that it is possible to make a retrospective diagnosis in a patient with scar formation. This can be done not only by using the serum diagnosis of Widal and Abrami (which lacks specificity), but above all through culture of the bucco-pharynx, since the fungus frequently remains saprophytic on the mucosae.

*Frequency of Occurrence.* The frequency of mycoses depends largely on the thorough and systematic search for them. In March, 1907, I had isolated four cases; by the end of 1907, thirty cases; by the end of 1908, sixty; and finally, by the end of 1910, more than two hundred. Observations have been reported from all French provinces, from all countries on the five continents, and in patients of all ages. Consequently, physicians in any country may come across sporotrichosis. The apparent rarity of the disease is due to the fact that it has become so classical that only observations which present interesting new details are reported. Nevertheless, sporotrichosis occurs all the time, as evidenced by three recent observations, namely, that of Banks<sup>6</sup> where a sporotrichosis gave the appearance of diphtheria, that of Pessano and Negroni,<sup>7</sup> and one case, described by Watrin and Didier,<sup>8</sup> of sporotrichosis of the leg.

All the same, the sporotrichoses have certainly decreased in frequency, at least in France, despite our systematic search for them. This seems also to apply to other continents; for example, R. O. Noojin and J. L. Callaway mention that they have found only seven cases in ten years. The question can be asked whether this is due to the extinction of all human and animal reservoirs by improved diagnosis and treatment with iodides. Infection

from human to animal and *vice versa*, is, of course, exceptional at best. In any case, there is no question of contamination by improperly sterilized syringes (certain specialists have advanced this bizarre theory), since in our well-conducted investigations we have never seen an "epidemic" and our patients never received any injections.

*Clinical Polymorphism.* In medical practice, my own observations, supplemented by those of other authors, have described numerous forms, ranging from a gumma and even large abscess to acne and sporotrichotic pityriasis.

Lesions may occur in all tissues. In the bones, joints, and synovia, sporotrichoses exhibit everything that can possibly be imagined, from periostitis to abscesses in the interior of the bone, spina ventosa, and spontaneous fractures, from simple hyarthrosis to white swelling, as well as various forms of synovitis. I have stressed the singular frequency of lesions in the bones, which is about 10 per cent in disseminated sporotrichoses—a number not reached by syphilis.

In the viscera, cases of orchio-epididymitis have already been reported, and also pulmonary congestion with hemoptysis,<sup>9</sup> and pyelonephritis (Rochard, Duval, and Bodoleo). There has been a recent report by André Martin of a renal sporotrichosis simulating the appearance of a sarcoma, later that of tuberculosis.<sup>10</sup> Further reports have been made of a febrile form (de Brissaud and Rathéry), and a fortunately exceptional, mortal one (de Roux-Lacroix; Banks). Sporotrichoses can simulate the appearance of blastomycoses (H. G. Adamson), actinomycoses (Gougerot), diphtheria (Banks), *etc.* As I have repeatedly stated, "The sporotrichoses, for all these reasons, are of interest not only to the dermatologist, but also to the internist, the surgeon, the oculist, obstetrician, pediatrician, and psychiatrist, as well as in forensic medicine."<sup>11</sup> It is necessary to make a diagnosis immediately without waiting until the patient becomes cachectic, because, at that stage, death is frequently inevitable, despite treatment with iodide. The prognosis, thus, truly depends on the scientific reliability of the examining physician, with the exception of certain rare cases with associated tuberculosis where the iodide treatment is impossible."

In practice, I have described and stressed several diagnostic phenomena, namely, the gummatous form which reappears upon incision and again reappears under the scar of the incision, disclosing the typical dome-shaped form with softening in the center; the polymorphism of the ulcerations (tubercular, syphilitic, or other in appearance), which may be evident either on different ulcers situated closely together, or on different sectors of the same ulcer; and the rapid relapse after premature cessation of the iodide treatment.

As regards general pathology, this group of observations shows that, as in bacterial infections, in particular those of tuberculosis and syphilis, one single virus can exhibit very different clinical forms, and that the same clinical phenomenon, as for example the gumma, may be due to different viruses or fungi.

*Histology.* The histological formula of the *Sporotricha* can be established



as consisting of three zones, namely, (1) the central zone formed of polynuclear cells and macrophages simulating the presence of a pyoderma; (2) median zone, tubercular in appearance, with epithelioid cells, giant cells, and tuberculoid follicles; and (3) the external zone, composed of lymphocytes and connective tissue cells, basophilic, with comparatively intense vascularity, frequently exhibiting almost a syphilitic sclerosis. These three reactions may even coexist without any order, *i.e.*, they may be mixed up particularly in the verrucous and vegetating sporotrichoses.

This histological reaction is nonspecific, since it is also encountered in other mycoses and even in the foreign body reaction of tissues, as my clinical observations have shown. In this connection, the experimental study by Vaucher and myself, especially with pepper, should be noted.

The histogenesis of the lesions is much less rapid than that of syphilis and tuberculosis, owing to the manner of the sporotrichotic process. Thus, we have been able to show, with a series of excised tissues, how the vascular stage is transformed into giant cells and, subsequently, into tuberculoid follicles and gummatous tissue with the three zones. This confirms the opinion of Gernil and other French authors who believe that the formation of giant cells and tuberculoid follicles is of vascular origin.

Pathologically, all these studies have shown once more that the sporotrichoses have no histological specificity, since they may exhibit pyococcic, tubercular, and syphilitic reactions.

*Etiology and Pathogenesis.* Through my clinical observations and experimental studies (particularly in collaboration with Vaucher), the etiology and pathogenesis of the sporotrichoses have been clarified inasmuch as they exhibit all possible clinical forms and are capable even of taking the shape of a bacterial infection. Their reservoir lies within the limits of nature (as for the blastomycoses, actinomycoses, *etc.*). I have demonstrated this by finding "wild" *Sporotrichum beurmanni* on an oat plant, a horsetail, and a small beech tree, in the French Alps in 1908. Upon cultivation, these wild specimens were not virulent; only after passing from rat to rat did they become pathogenic. Soon afterwards, Sartory<sup>12</sup> found the same *Sporotrichum* on an ear of wheat; the first generation was non-pathogenic, but, after passage through several animals, the organism became virulent.

Animals are but rarely the source of contagion, and they themselves are generally infected by plants. Pertinent observations here have been recorded by Lutz and Splendoré, as well as by Jeanselme and Paul Chevalier (rat sporotrichosis transmitted to man); Page, Frothingham, and Paige, also Mervins-Hyde and Davis, and Mohler (horse sporotrichosis); R. L. Sutton (human contamination); T. C. Jones and Fred D. Maurès (mule sporotrichosis—see especially Carougeau's case of contamination of the veterinarian treating an affected mule); Gougerot and Caraven (dog sporotrichosis); Wyss-Lauzin (parrot sporotrichosis, and transmission to the human by a bite); and Olson (rodent sporotrichosis, with transmission to man).

The same phenomena are found here as in tuberculosis, such as primary chancre at the site of infection (see my observation No. 12) or lymphangitis

without visible portal of entrance (No. 13); saprophytism on the mucous membranes and germ carriers (J. Thiry); infection through the intestinal canal; and dissemination through the lymphatics or the blood circulation (Widal and Weill). The same factor of lowered resistance accompanied by progressive adaptation and virulence as in diabetes and tuberculosis, *etc.*, has been demonstrated on the sporotricha found in nature. Increasing sensitization in man has been elicited by the intradermal reactions reported by Paul Ravaut, Verdin, and myself, and thus the importance of the human reservoir has been stressed. Also, hybrid infections, such as combinations of sporotrichosis and tuberculosis, have been encountered (Achard and Louis Ramond: personal observations).

*Experimental Reproduction.* As outlined in the foregoing, our long experimental studies have clarified the clinical treatment, the histology, etiology, and pathogenesis of sporotrichosis. However, experimentation has taken the lead from clinical studies as regards the visceral forms, which give a picture of the bone infections and other forms. With Vaucher, I have even outlined a possible hereditary sporotrichosis, thus again demonstrating the similarity to bacterial infections and syphilis.

*Treatment.* The iodide treatment has been patiently evolved and regulated according to the four well-known categories of patients: (1) tolerant to iodide; (2) partially intolerant to iodide; (3) completely intolerant to iodide; and (4) local lesions not responsive to iodide. I have stressed the following rules for this treatment: doses of 4 gm. daily; necessity for supervising the tolerance of iodide, which may give a clue to possible latent tuberculosis; necessity of prolonging treatment at least one month beyond the apparent healing of the lesions, since relapses frequently occur otherwise; consolidating treatment; observation of a possible pharyngeal reservoir of parasites; and recognition of the gravity of cases associated with tuberculosis.

I have made studies parallel to the work of Achard and Louis Ramond, proving that the iodide treatment is not effective through direct antiseptic action, since it is possible to cultivate the sporotrichosis fungi *in vitro* in from moderately to very strong iodide solutions. Rather, the iodide acts through stimulation of the macrophages and of the defense mechanism of the tissues.

Recent research, particularly in the United States, has shown decided effects of the sulfonamides. R. O. Noojin and J. L. Callaway, for example, used sulfonamide and sodium salt of sulfapyridine, in local treatment, with a 5 per cent oily suspension.

### Conclusion

Through all the studies enumerated in this paper, the sporotrichoses have been classified, pathologically, among the infections, *i. e.*, they have been separated from other mycoses. The study of mycoses has thus been given new vigor, and it is no longer necessary to contrast them against bacterial infections, as has been done in the past, but rather, connections between the two can be established.

The study of the sporotrichoses, through systematic research in all the mycoses, has led to the discovery of several new forms as, for example, hemisporosis, caused by *Hemispora stellata* (Gougerot and Caravell); oidiomycosis or mycodermosis, caused by *Oidium* or *Mycoderma culaneum* (Gougerot, de Beurmann, and Vaucher); nocardiosis of Carougeau; gummatous parendomycosis, caused by *Parendomyces balzeri*, and mycodermosis caused by *Mycoderma pulmoneum* (Gougerot, Balzer, and Burnier). In collaboration with Duché,<sup>13</sup> I found disseminated polyepidermomycosis of the intertriginous regions (leaving their basis intact), with the presence of *Epidermophyton inguinale*, *Malassezia furfur*, and a new fungus, *Aspergillus endovici*; also, a new subcutaneous mycosis, caused by *Debarcomyces klockeri*. Finally, with Burnier and Duché, I discovered a vegetating and ulcerative mycosis, caused by *Cephalosporium griseum*.

The classical clinical analysis of the sporotrichoses was made by my old teacher, Louis Landousy,<sup>14</sup> who stressed the practical prognostic, therapeutic, and economic importance of the sporotrichoses. Time has indeed passed since another of my great teachers accused me of being a youthful hothead "trespassing on the sacred ground of syphilis and tuberculosis." I have repeatedly drawn attention to the faulty diagnosis of tuberculosis, syphilis, chronic osteomyelitis, glanders, and even cancer, which has led to amputation and death, and I can but once again repeat the words of Professor Grasset, the famous neurologist: "A physician who is not familiar with the latest research on sporotrichosis runs the risk of letting patients die who could have been saved by the judicious administration of potassium iodide."

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# GROWTH REQUIREMENTS OF DERMATOPHYTES

By William J. Robbins

*Department of Botany, Columbia University, and Director, New York Botanical Garden*

It is not my intention to discuss the growth requirements of the dermatophytes in general, but to confine my attention chiefly to two with which we have been mainly concerned. One of these is *Trichophyton discoides*; the other is *Trichophyton mentagrophytes*.

The strain of *T. discoides* with which we worked was obtained from Dr. Juan Mackinnon of the Institute of Hygiene, Montevideo, Uruguay. It was found that for growth this isolation required the presence of molecular thiamine, pyridoxine, and inositol in the medium. It failed to grow on a medium which was limited to dextrose, mineral salts, and asparagine. The addition of thiamine, pyridoxine, and inositol to this basal medium permitted considerable growth to occur. All three vitamins were necessary. Little or no improvement in growth was obtained with the addition of any one of the three or any two of the three.

The thiamine deficiency could not be satisfied by the pyrimidine component of thiamine, by thiazole, or by a mixture of the two thiamine intermediates. Neither calcium phytate nor a phosphatide containing inositol obtained from Dr. D. W. Woolley were as satisfactory as inositol. The phosphatide substituted for inositol more nearly than did the calcium phytate.

The minimum effective quantities of the three vitamins were not determined, but the addition of 0.01 mg. of inositol to 8 ml. of medium which contained both thiamine and pyridoxine produced a marked effect on growth. The maximal effect was obtained with between 0.1 and 0.5 mg. of inositol. The addition of 0.001 m $\mu$  mole of pyridoxine to 8 ml. of medium containing thiamine and inositol was ineffective, but 0.01 m $\mu$  mole produced a measurable effect. Maximum growth was obtained with between 1 and 10 m $\mu$  moles of pyridoxine. The addition of 0.01 m $\mu$  mole of thiamine to 8 ml. of medium containing pyridoxine and inositol markedly increased growth, and the maximum effect was obtained with between 1 and 10 m $\mu$  moles of thiamine.

Neopeptone in the amounts we used did not contain sufficient thiamine for maximum growth. Better growth was obtained when the mineral-dextrose-peptone medium was supplemented with thiamine. Inositol or a physiological equivalent material was found to be present in hydrolyzed gelatin, hydrolyzed casein, hydrolyzed egg albumen, and peptone, as evidenced by the growth of the organism on these media supplemented with thiamine and pyridoxine but no inositol.

Although it was possible to obtain considerable growth on the basal medium of mineral salts, dextrose, and asparagine, supplemented with thiamine, pyridoxine, and inositol, better growth was obtained on a thiamine peptone medium or on gelatin hydrolysate or casein hydrolysate supplemented with the necessary vitamins. It appeared, therefore, that gelatin

hydrolysate, casein hydrolysate, and peptone contained unidentified factors important in the growth of *T. discoides*. Although we were not successful in duplicating the effect of the protein hydrolysates by the substitution for them of a mixture of amino acids and purine bases, it is probable that the action of the hydrolysates was an amino acid effect.

Our investigations, therefore, showed that the strain of *T. discoides* which we used evidenced complete deficiencies for molecular thiamine, pyridoxine, and inositol and partial deficiencies for unidentified growth substances present in peptone and hydrolysates of gelatin or egg albumen. The unidentified factors are probably amino acids.

The strain of *T. mentagrophytes* that we investigated was isolated from a human subject and identified through the courtesy of Dr. Rhoda W. Benham. This organism grows slowly on a basal medium containing mineral salts, dextrose, and asparagine. Its growth is markedly improved by the addition to the basal medium of peptone, or hydrolysates of casein, egg albumen, or gelatin.

In contrast to *T. discoides*, the isolation of this organism has no vitamin deficiencies. No improvement in growth has been observed from the addition of vitamins to the basal medium or to the basal medium supplemented with vitamin-free protein hydrolysates.

Since this fungus evidenced no deficiencies for any of the known vitamins and its growth was markedly improved by hydrolysates of egg albumen or highly purified gelatin, and of vitamin-free casein, it seemed probable that the active substances were amino acids. It was found that the organism would grow on a basal medium containing asparagine or any one of the following 14 amino acids: glycine, dl-alanine, dl-valine, l-leucine, d-isoleucine, dl-phenylalanine, d-glutamic acid, dl-aspartic acid, dl-serine, l-cystine HCL, l-tyrosine, l-proline, d-arginine HCL, and l-histidine HCL. Only 5 of 19 amino acids tested gave little or no improvement in growth. These were tryptophane, threonine, hydroxyproline, methionine, and lysine. Ammonium nitrate was nearly or completely unavailable. However, we were not able to duplicate completely the effects of casein hydrolysate, for example, by any mixture of amino acids we prepared.

Our strain of *T. mentagrophytes* was able to use asparagine or any one of 14 amino acids as a source of nitrogen, but was nearly or completely unable to utilize ammonia or nitrate nitrogen. It appeared able, therefore, to transform asparagine or any one of the several amino acids without previous ammonification into all the various amino acids required for the construction of its protoplasmic proteins. We found no evidence for any indispensable amino acid in the sense that the fungus failed to grow unless a particular amino acid was furnished in the nutrient medium. Although *T. mentagrophytes* is capable of making all the amino acids needed for its proteins from a single amino acid or from asparagine, a mixture of certain amino acids was superior to an equal amount of any single one. This, we believe, is because the amino acids, if available in the medium, are incorporated into fungus protein more rapidly than they are supplied by the metabolic transformation of asparagine or of a single amino acid. In other words, the

mechanism for nitrogen transformation in this organism is deficient. It lacks, nearly or completely, the machinery necessary for utilizing inorganic nitrogen. Also, its mechanism for transforming asparagine or a single amino acid into those required for the construction of cell substance works slowly.

One of the complicating factors in the study of the nutrition of the dermatophytes is the freedom with which many of them produce pleomorphic forms which may differ in their physiology from the organism from which they are derived. Pleomorphic forms have long been recognized in this group. They are spontaneous irreversible mutations.

We have investigated chiefly the pleomorphic forms of *T. mentagrophytes*. These mutants develop as cultures of this organism age. Portions of the mycelium change in growth habit. Many pleomorphic types can be isolated which differ in rate of growth, degree of sterility, morphological character of the colony, pigment production, or some physiological character. All cultures, if sufficiently old, are characterized, however, by a vigorous, white, more or less fluffy, almost sterile form. The growth of the most vigorous forms is many times that of the original isolate. In a liquid medium containing asparagine as a nitrogen source, the normal form produces three or four milligrams of dry matter in the course of three weeks. A pleomorphic form, in the same time in the same medium, may form a hundred milligrams or more.

We have found it possible to maintain the slow-growing, freely sporulating form, which we call the normal or N form, by making transfers to fresh media at weekly intervals. By such a procedure, we have kept *T. mentagrophytes* for more than four years, apparently unchanged. The pleomorphic forms, too, can be isolated, grown in pure culture, and maintained if they are transferred at weekly intervals.

The spontaneous change from normal to pleomorphic occasionally appears in cultures incubated at 35° C. as early as eight days after inoculation, though two or three weeks are usually required, and within four or five weeks at most all cultures will have become pleomorphic. It is not, therefore, a hit-or-miss process. Merely by allowing the cultures to age, all of them will change from a slow-growing, freely sporulating form to one which grows rapidly and is nearly or completely sterile.

From the standpoint of this discussion, these pleomorphic strains are important because they differ in their growth requirements from the original form from which they are derived. For example, some of the rapidly growing mutants of *T. mentagrophytes* are able to use inorganic nitrogen, which is nearly or completely unavailable to the normal form (N) from which they arose. The pleomorphic forms are characterized by a greater ability to transform ammonium salts and also asparagine into those amino acids necessary for the construction of their protoplasmic proteins. These transformations are probably enzymatic, which means that in becoming pleomorphic the fungus has developed new or more effective enzyme systems than exist in the normal form.

I do not mean that the only difference between the normal and pleo-

morphic strains is a change in the ability of the organism to transform a single source of nitrogen into the various amino acids required for the construction of cell substance. I should expect to find that pleomorphic forms of a dermatophyte which has a specific vitamin deficiency might be able to synthesize the vitamin which the normal form is unable to make. We have found this to occur in pleomorphic forms (sectorial mutants) of *Fusarium avanaceum*. The original isolation lacked the power to synthesize biotin, but a vegetative mutant developing spontaneously from the original isolation was able to make its own biotin.

One of the peculiarities of the dermatophytes is their relation to hydroxyproline. This amino acid, a common constituent of proteins, was found to inhibit the growth of five dermatophytes investigated, but to have no comparable effect on 19 other fungi, including species of *Agaricus*, *Aspergillus*, *Ceratostomella*, *Fomes*, *Neurospora*, *Penicillium*, *Phycomyces*, *Polyporus*, and *Rhodotorula*.

The growth of *T. mentagrophytes* on a basal medium containing mineral salts, dextrose, and asparagine, was almost completely inhibited by one part of l-hydroxyproline in 20,000 parts of medium, and a reduction in growth was observed with one part in 50,000 parts of medium. Two pleomorphic forms of *T. mentagrophytes* proved to be more resistant to hydroxyproline. One of them required one part of hydroxyproline in 1600 parts of medium, and the other, one part in 4000 for complete or nearly complete inhibition. We observed also the development of a strain of this organism which was quite resistant to hydroxyproline. Good growth was obtained on a basal medium to which one part of hydroxyproline was added to 160 parts of medium.

*Trichophyton purpureum*, *Microsporum canis*, *Epidermophyton flocculosum*, and a granular form of *Trichophyton gypsum* were also susceptible to the injurious effects of hydroxyproline. The *Epidermophyton* was the most sensitive. Almost complete inhibition was obtained on the addition of one part of hydroxyproline to 40,000 parts of the basal medium. *Microsporum canis* was the most resistant. The growth of this fungus was reduced but not prevented by one part of the hydroxyproline in 1600 parts of medium.

In contrast to the effect of this amino acid on the dermatophytes was its action on such organisms as *Penicillium notatum*, *Rhizopus nigricans*, and *Fomes pini*. These fungi showed no reduction in growth even in the presence of one part of hydroxyproline in 1600 parts of medium.

The injurious effect of hydroxyproline on the dermatophytes was less pronounced in media containing peptone or casein hydrolysate than in those containing asparagine. The effect of each of 14 amino acids on the inhibitory action of hydroxyproline was determined for the normal form of *T. mentagrophytes*. Proline alone was found to overcome, at least in part, the injurious effects of hydroxyproline. There seemed to be, therefore, some evidence for an antagonism between l-proline and l-hydroxyproline.

One of the curious responses to hydroxyproline was observed with *T. purpureum*. The growth of this organism was inhibited almost completely

by one part of hydroxyproline in 1600 parts of medium. Also, a marked reduction in growth was obtained with one part of the amino acid in 4000 parts of medium. With small amounts, one part in 40,000 or one part in 20,000, growth was stimulated. The colonies obtained were larger than observed on the basal medium.

The effects of hydroxyproline on the dermatophytes is interesting because it seems to be evidenced on these organisms as a group, which suggests a common physiological characteristic. It is also of interest because proline is an available source of nitrogen for these organisms and the only difference between proline and hydroxyproline is a hydroxyl radical in the fourth position on the molecule.

A single and relatively simple explanation of the relation of hydroxyproline to the growth of fungi does not seem possible. The action of hydroxyproline may be associated in some way with the nitrogen metabolism of these organisms. This is suggested by the greater sensitivity of the normal form of *T. mentagrophytes* as compared to the pleomorphic forms. The latter, which are the more resistant, are also better able to supply their nitrogen requirements from inorganic nitrogen or from a single source of organic nitrogen.

This has been only a fragmentary presentation of the growth requirements of dermatophytes. There has been no discussion of their relation to hydrogen ion concentration, to reduced sulphur, or to carbon sources or many other of the fundamental nutritional factors which should be considered. There has been no attempt to review here the contributions made by others to this subject. The paper has been limited to the specific researches with which the author and his associates, Dr. Roberta Ma and Dr. Ilda McVeigh, have been concerned.

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# FUNGUS ANTIGENS AND THEIR IMPORTANCE AS SENSITIZERS IN THE GENERAL POPULATION

By Samuel M. Peck

*Mount Sinai Hospital, New York, N. Y.*

Disease manifestations caused by both bacteria and fungi can be divided into those which are directly due to these organisms and those special forms which have arisen because of the development of sensitization to the organisms and/or their products. The latter have been grouped under the general heading of cutaneous microbids.<sup>1</sup>

The clinical manifestations of microbids depend on a development of an acquired hypersensitivity to the organisms and/or their products after the primary infection has existed for some time. The degree of acquired hypersensitivity is dependent on the causative organism, on individual predisposition, and on many other factors which cause more intimate contact between the living organisms and the living cells.

In the group of microbids, we have trichophytids when the trichophyton fungus is the primary cause of the lesion, epidermophytids when an epidermophyton is the causative organism, and levurids when monilia cause the primary infection. Trichophytid is the general term which has been applied to the microbid associated with fungus infections. The term in the literature has often been shortened to "ids."

The allergic manifestations due to fungi which are most commonly encountered in the general population are those associated with the superficial fungus infections. Of these, almost all are associated with infection due to *T. mentagrophytes*, especially dermatophytosis of the feet.

## *Pathogenesis of Trichophytids*

The *Epidermophyton* and *Trichophyton* fungus grow in the nonliving layers of the skin and its appendages. Because of this fact, they give rise to clinical manifestations which are primarily of a very superficial nature. Marked inflammation does not develop unless living structures are invaded. Invasion by the fungi of living structures or contact of fungi or their products with the living parts of the skin probably initiate the hypersensitivity with its resulting allergic manifestations. W. Jadassohn and Sulzberger have shown in experimental fungus infections in guinea pigs that the apparent dermatotropism (keratinotropism) of fungi depended on their need for dead tissue such as is found in the keratin layers of the skin, the hair shaft, and the nails. When they injected guinea pigs intracardially with fungus spores, there was a hematogenous dissemination all over the body, but fungus infection developed only in the nonliving parts of the skin. When the animal was killed shortly after injection, however, and the internal organs were cultured, fungi readily grew from this dead tissue. It is of interest to note that the lens of the eye (Jadassohn-Rehsteiner) could be infected with fungi just like the keratin layers of the skin.

A *sine qua non* for the development of trichophytids is a hypersensitivity

to fungi or their products. This sensitivity is revealed by a positive trichophytin reaction. Fortunately, the trichophytin test represents a group reaction in the majority of instances. This means that the test does not usually have to be made with the identical fungus causing the lesions. Previously it was thought to be present only in the deep inflammatory fungus infections, such as *kerion celsi*. Recently, however, it has been demonstrated that even superficial fungus diseases were frequently accompanied by sensitivity to trichophytin. The hypersensitivity to fungi develops after the primary infection has existed for some time. The period between onset of infection and the development of hypersensitivity varies from a few months to several years, depending on the type of fungus, the site of the primary infection, and the infected individual. It is very difficult to determine whether atopy plays a role in the development of this hypersensitivity. In the available statistical data, a history of atopy seems rather unimportant, especially as far as the epidermophytids on the hands are concerned.

Trauma, such as treatment with strong ointments, X rays, or continuous maceration, which is frequently seen between the toes, forces the fungi or perhaps their products into the blood stream. These, in turn, coming in contact with the hypersensitive skin, give rise to trichophytids. The localization of the trichophytids may depend on the anatomy of the circulation or other rather obscure moments. The work of Truffi with fungi and of W. Jadassohn with tuberculosis has shown that localization can be determined experimentally by causing rupture of capillaries, thus allowing the circulating noxa to become free and reach the skin. The circulating organisms are changed by immune bodies in the blood and reach the skin in an attenuated state. To reach the keratin layer (*i.e.*, the dead tissue in which they can grow), they pass through the living structures which have become hypersensitive. The resulting interaction destroys the majority of them and gives rise to the trichophytids. If any organisms pass through these barriers and reach the dead tissues, they can be demonstrated in the trichophytids. This is rather a rarity.

If we were to assume that not only the fungi but also their toxins can give rise to trichophytids, it would be easy to understand why trichophytids are usually sterile. Such an assumption, however, would not explain the localization of epidermophytids on the hands only, secondary to the fungus infection of the feet in the presence of a generalized skin sensitivity. Furthermore, it has not been possible to demonstrate such circulating toxins while positive blood cultures for fungi identical with those causing the primary infection have been obtained.

Williams has pointed out that, once hypersensitivity has been established, even the primary lesion is a combination of epidermophytosis and epidermophytids. This explains the difficulty very often encountered in demonstrating fungi from such areas. It is obvious that scrapings from trichophytids will give negative microscopic results.

It is within the realm of theoretical possibilities that contact with primary lesions can cause transportation of the organisms to other parts of

the body and give rise to trichophytids by contact. The eruptive character of most of the microbids speaks against such a conception. Such a possibility must be borne in mind, however, when nonsymmetrical isolated lesions are found which are considered to be trichophytids, especially those in which the organisms are more readily demonstrated.

It is even conceivable that organisms of great virulence can reach areas of skin hematogenously and, in the presence of a moderate degree of hypersensitivity, win through all barriers to give rise to lesions which are real secondary foci of epidermophytosis (trichophytosis). Since the attention of dermatologists has been focused on the microbids, this diagnosis is being made with great frequency. Very often the proper supporting evidence for such a conception is lacking. Experimental and clinical research has established certain definite criteria which must be present to support such a diagnosis. Such criteria are as follows:

(1) The causative organism must be demonstrated in what is recognized by every one as a classical manifestation of the disease.

(2) While it is not absolutely essential, the organism which is cultured from the primary lesion should be pathogenic.

(3) A positive reaction analogous to a tuberculin or a trichophytin reaction must be present.

(4) What is considered to be microbid should be seen as a frequent accompaniment of the primary lesion.

(5) Positive blood cultures for the same organism isolated from the primary lesion must be obtained, since it is admitted that most of the microbids are hematogenous eruptions. This is necessary because there is no reliable method of demonstrating the presence of circulating toxins.

(6) The microbids must develop subsequent to the primary infection.

(7) The microbids must usually be sterile.

(8) A support for the conception of a skin eruption as an "id" lies in certain clinical characteristics: (a) appearance of the "ids" in showers; (b) tendency to symmetry in distribution because of hematogenous origin; (c) tendency to spontaneous involution after healing of the primary focus; (d) focal reactions after injection of sufficient amounts of microbidin.\*

It has been shown that the trichophytids will appear in an individual having a focus of fungus infection provided that hypersensitivity to the fungi or their products developed. After the hematogenous dissemination of the organism or their products, the allergic manifestations will take place in that organ which is the particular shock tissue. In the case of fungus infections such as trichophytosis or epidermophytosis, the skin is that particular organ.

While it is true, as is the rule for other microbic diseases, that different fungi can elicit the same clinical picture and that totally different skin manifestations can be found associated with the same fungus, it was found, fortunately, that in the majority of instances we could associate the various types of trichophytids with certain of the primary fungus diseases. Thus,

\* Microbidin: I must apologize for the introduction of a new term in a branch of medicine so overloaded with names, but it is only proper to give this general term to the extract or vaccine which denotes skin sensitivity in cases of microbids.

the *Lichen trichophyticus* usually accompanied markedly inflammatory fungus infections such as kerion celsi (trichophytosis), while the dyshydrotic eruptions of the hands were usually found associated with the epidermophyton infection of the feet.

The localization of the embolized fungi and the site of greatest skin sensitivity play an important part in the morphology of the resulting trichophytid. If the organisms finally become localized in the vasa vasorum of the subcutis, a subcutaneous trichophytid (*erythema nodosum*) develops; if they become localized in the vessels of the hair follicle, lichenoid forms result; if the epidermis is a special site of sensitivity, eczematoid trichophytids are found; and, if the hypersensitive organism is flooded with toxins, diffuse scarlatiniform eruptions are seen.

TABLE 1

## TYPES OF TRICHOPHYTIDS

- I. Epidermal trichophytids (epidermis mainly involved)
  1. Eczematoid (dyshydrotic)
  2. Lichenoid
  3. Parakeratotic
  4. Psoriasiform
- II. Cutaneous trichophytids (papillary body mostly involved)
  1. Diffuse forms
    - a. Scarlatiniform exanthemata and enanthemata
    - b. Erythroderma
  2. Circumscribed and disseminated forms
    - a. Follicular localizations usually lichenoid
    - b. Not exclusively follicular
      - (1) macular, papular, and even exudative eruptions
    - c. Erysipeloid
- III. Subcutaneous trichophytids (nodules found in the hypoderm of the type of erythema nodosum)
  1. Acute resolving form
  2. Destructive chronic form
- IV. Vascular trichophytids
  1. Migrating phlebitis (venous)
  2. Urticaria (capillary)
  3. Purpuric

As can be seen from TABLE 1, the trichophytids can be classified under four headings, depending on their histologic and clinical characteristics. The epidermal and cutaneous types are the most common. The lesions under Group IV have only lately been recognized as trichophytids, and I have observed several cases in which the recurrent phlebitis seems to be accompanied by other vascular sensitivities, even epileptiform seizures. No doubt the most important and frequent trichophytid observed is that accompanying epidermophytosis. The epidermophytosis is usually on the feet, while the epidermophytids are on the hands. The essential proofs for their relationship, as laid down in previous paragraphs, have been well established in this important group by the work of Peck and W. Jadassohn. Even such difficult evidence as positive blood cultures for fungi have been presented by Peck and Strickler, Ozellers, and Zaletel. Furthermore, Peck has been able to reproduce the whole clinical syndrome experimentally in

humans. This was the first time that a spontaneous experimental trichophytid had been reproduced in a human subject.

While the dyshydrotic trichophytids and the type known as dyshydrosis *Lamellosa sicca* are fairly easy to diagnose, the eczematoid trichophytids, especially when they occur on the hands, are often impossible to differentiate from ordinary eczema. It is just in this group that failures of diagnosis occur, because of the great prevalence of epidermophytosis of the feet, with its accompanying positive trichophytin reaction. This difficulty of diagnosis and the need for methods of differentiation is well recognized by all workers in the field. It is for this reason that serological criteria for diagnosis have been attempted.

Recent literature, both foreign and American, has become increasingly filled with the discussion of levurids: those mycids which are due to monilia. Ravaut, a pupil of Sabouraud, described the first cases. Very interesting examples of such eruptions have been demonstrated by Ramel, Hopkins, and others. As Bloch has pointed out, in discussing the research of Staehelin from his Institute, it is very difficult to prove that an eruption is a levurid. One of the chief stumbling blocks seems to be to prove a pathogenic role for monilia cultured from what is called the primary lesions. It is difficult to be certain that the reaction following the intradermal injection of oidiomycin is based on true hypersensitivity because of its practically one hundred per cent incidence in the adult. The last, however, cannot be considered as very strong negative evidence when we consider that the number of the oidiomycin reactions increases with the age group and that, in certain classes of the population, even the tuberculin reaction may approach one hundred per cent incidence. However, one of the most important steps in the evidence necessary to prove the existence of levurids, namely, positive blood cultures taken under rigid control conditions, is lacking.

Nevertheless, the question of the role of monilia and related organisms as secondary invaders in fungus infections, eczema, and other skin conditions must be considered, especially if we are to place any value on a positive oidiomycin reaction.

#### *Incidence of Dermatophytosis in the General Population*

When the incidence of dermatophytosis in the general population was discussed, until the recent epidemic of tinea capitis, the discussion was mainly concerned with fungus infections of the feet, hands, and groins. A number of authors have investigated this problem. The largest group of cases included in one study was that carried out by the Public Health Service in 1943-1944.<sup>2</sup> Approximately 2100 people living in the District of Columbia, New Jersey, Connecticut, Indiana, and Louisiana were included in the pH survey. The effects of different seasons on the incidence of dermatophytosis also were studied. The fungus infections encountered rarely affected parts of the body other than the hands and feet. In that study, 1393 men and 733 women were examined. Their ages varied from 17 to 70 years, but most of those examined were in the second and third

decade. Based on clinical and cultural grounds the classification of the examinees were as follows: positive 590 (27.79 per cent); doubtful 714 (33.63 per cent), and negative 819 (38.57 per cent). Classified according to sex, of 1393 men, 391 (28.06 per cent) were in the positive group, 500 (35.89 per cent) in the doubtful group, and 502 (36.03 per cent) in the negative group. Of 733 women, 199 (27.2 per cent) were in the positive group, 214 (29.31 per cent) were in the doubtful group, and 317 (43.42 per cent) were in the negative group.

It was shown by cultural study that some patients in the clinically doubtful group had true dermatophytosis. There was no significant difference in sex incidence. When the same personnel were examined at different seasons, the number of clinically negative patients rose from 13 per cent to 19 per cent in the summer to 54 per cent in the winter. Thus it can be seen that there is a definite influence of seasons as far as clinical evidence of the activity of fungus infections is concerned. The most frequent pathogenic fungus recovered was *T. gypseum*. *T. purpureum* was next in frequency and *E. inguinale* was only recovered from an occasional case. All of the cultures of *T. purpureum* were recovered in one locality.

The work of Osborn and Hitchcock<sup>3</sup> seems to show that the women are less affected with dermatophytosis than the men. The Public Health Service surveys, however, did not show any significant difference in sex incidence.

During the war it was found that 8 per cent of all hospital admissions in the Army and the Navy were for cutaneous disease and that dermatophytosis ran second on the list.

According to Weidman and his associates<sup>4</sup>, the estimates of Peck *et al.*<sup>3</sup> of the incidence of clinically active dermatophytosis of the feet is conservative. They maintain that approximately 65 per cent of the population is affected. Children below the age of 10 on the whole have a very low incidence of foot mycoses.

Scalp ringworm has always been endemic in a small percentage of the children in the country. Early in 1942, however, it became apparent that infection of the scalp with fungi was rapidly assuming epidemic proportions. By 1945 the epidemic, which first started in large Eastern centers, rapidly swept westward and it is now nationwide. It is well known that at or shortly after puberty tinea of the scalp, as it is commonly seen, disappears spontaneously.

Thousands of cases of tinea capitis have been examined in the last few years and the causative fungus isolated. Most observers have noted that *Microsporon auduini* and *Microsporon lanosum* were responsible for nearly all of the cases of scalp ringworm. However, the majority of observers agree that all but a few of the cases in the present epidemic were found to be infected by *M. auduini*. The scalp ringworm due to a microsporon of animal type like *M. lanosum* is associated with a large proportion of sensitization to the fungus. The human type of microsporon like *M. auduini* is much more resistant to local therapy because of the lack of accompanying sensitization.

In a recent study<sup>2</sup> of the 6598 pupils of grade and junior high schools in Hagerstown, approximately 8.3 per cent were discovered to have ringworm of the scalp, nearly all due to *M. auduini*. During the period of the study, August 1944 to November 1945, the number of boys infected was 12.1 per cent of the boys examined; the number of girls infected was 2.1 per cent of the number of girls examined. Most of those affected were under 12 years of age.

### *Incidence of Trichophytin Sensitivity*

To obtain an idea of fungus allergy in the general population, the trichophytin test was performed on 776 persons living in different sections of the country. Of these, 57.47 per cent could be classified as negative and 42.53 per cent had a reaction which varied from one to four plus.

It was interesting to note that, of 558 males tested, 48 per cent showed a positive reaction. Of 218 females tested, 29.96 per cent showed a positive reaction. This was of interest because it was found that there were practically as many women as men with clinically positive evidence of fungus infection of the feet. The statement is frequently made that a positive trichophytin reaction is only an indication that the patient has once had a fungus infection and gives little information about the present activity of the infection. Our analysis of the results of the trichophytin test indicates that the trichophytin test shows a greater number of positive reactions in patients with active fungus infection of the feet than those who were classified as clinically negative. To some extent, the degree of reaction seems to bear a relation to the clinical activity of the infection. Lewis and Hopper found that 60 per cent of their patients with *T. gypsum* infection of the feet reacted to trichophytin. The number of trichophytin reactions rose to 87 per cent when definite clinical activity was present.

In a recent study by Peck *et al.*,<sup>7</sup> 406 adults living in and around New York were tested with trichophytin. Of these, 27.4 per cent were found to be positive. There were 250 males, with 36.9 per cent positive reactions, and 156 females, who had 14.2 per cent positive reactions. Also, 101 children under 12 years of age were tested with trichophytin and there was no positive trichophytin test in any of these cases.

### *Nature of the Trichophytin Antigens*

Trichophytin may be defined as an extract of fungi which is used both for diagnosis and treatment. It has been firmly established by many investigators, since the original preparation of trichophytin in 1902 by Plato and Neisser, that the positive reaction following the intracutaneous administration of this substance is due to a specific sensitivity resulting from a fungus—that is, a trichophyton infection.

Fungi of the *Epidermophyton*, *Trichophyton*, and other genera contain a general sensitizing factor, so that a patient infected with a *Trichophyton*, in whom a hypersensitivity has developed, will show a positive trichophytin reaction with an extract made from any of these organisms. According to Jadassohn, Schaaf, and Laetsch<sup>8</sup> there is an additional specie-specific excit-

ant which may not be present in any other members of the group. Because the reaction of sensitivity may be limited to this specific excitant, one may occasionally obtain a negative reaction to a trichophylin test even in the presence of ids, if the specific trichophylin used lacks this substance.

Trichophylin, as it is commercially available, is a complex material, probably containing a number of different antigens, the potency of which is dependent to a great extent on the method of preparation.<sup>9</sup> Therefore, it is of prime importance that, before it is used, some understanding be obtained of the method of preparation of the particular extract used for testing or treatment.

The usual method of preparation of trichophylin consists of inoculation of Sabouraud's bouillon with *T. gypseum* or *T. interdigitale* contained in a large Erlenmeyer or Roux flask at a pH of about 6.5 to 7. Growth is allowed to proceed at room temperature for 10 to 12 weeks until a large surface pellicle is formed. The pellicle is then tricherated with sand in a mortar after preliminary freezing with solid carbon dioxide. This freezing facilitates the breaking up of the mass of mycelium and spores. A sufficient amount of the bouillon is added to make a sludge. The semiliquid mass is placed in a shaking machine for 24 hours. It is then kept in an incubator for another 24 hours and again shaken for 24 hours. The mass of growth and broth is filtered through several thicknesses of filter paper and the filter is passed through a Seitz filter. The final filtrate may have to be diluted before use in order to avoid nonspecific reactions. It has been used for cutaneous tests and for treatment. Usually, 0.5 per cent phenol is added as a preservative.

There are 3 commercial fungus extracts available which are all called trichophylin. They are obtained by different methods of manufacture and are therefore not similar either quantitatively or qualitatively. It is obvious that since we lack a method of standardization the results obtained by the use of these different extracts are not comparable.

Bloch, Labouchere, and Schaaf,<sup>10</sup> in 1925, isolated from ordinary trichophylin a starch-like protein-free polysaccharide. Nitrogen, however, was still present after purification. These authors were able to demonstrate that this polysaccharide will still elicit positive reactions in patients sensitive to ordinary trichophylin. In addition, they demonstrated that it produced specific cutaneous reactions in guinea pigs infected by fungi and elicited a Shultz-Dale reaction in the uterine strip of guinea pigs previously infected with fungi. These experiments indicate that most likely the polysaccharide was an active and specific principle of trichophylin.

Da Fonseca and his collaborators<sup>11</sup> prepared what they called a classo-vaccine from over 300 strains of fungi, including *Trichophyton*, *Microsporon*, *Epidermophyton*, and *Endodermophyton*. This was protein-free and seemed to consist of a carbohydrate which closely resembled the preparation of Bloch. The Classo extract was said to be efficacious in treatment of many kinds of fungus infections, but it did not contain the skin-reactive substance capable of eliciting a positive reaction to a trichophylin test. Sulzberger, Lewis, and Wise<sup>12</sup> tested the Classo extract in a large series of patients and



in approximately 800 industrial workers and its relation to occupational dermatoses. Of these workers, approximately 43 per cent showed a positive trichophytin test, indicating sensitivity to fungi and/or their products. Among these cases, however, there were so few cases of allergic dermatoses that no conclusions could be drawn as to the relationship of trichophytin sensitivity to allergic dermatitis. However, when the incidence of positive trichophytin tests was studied among a group of cases with contact allergic dermatitis, it was found to be no greater than that among the general industrial population. If trichophytin sensitization as such played a role in the development of allergic contact dermatitis, the number of positive trichophytin cases should have been higher in this group than in those who did not have a sensitivity to the materials which they contacted.

It could be concluded from these studies that fungus infections play a minor role as causes of absenteeism, and sensitivity to fungi, while it is of frequent occurrence, plays no important role as a predisposing factor to the acquisition of other allergic contact dermatoses.

#### *The Relationship between Fungus Infections and Penicillin Sensitivity*

So far it is apparent that fungus antigens play an important role in eliciting allergic manifestations in the general population both because of the high incidence of fungus infection and because a relatively large number of those infected acquire a sensitivity to the fungi and/or their products. With the introduction of the antibiotics into therapeutics, however, the importance of the fungus antigens as causes of allergic reaction has immeasurably increased.

The clinical reactions to penicillin are of two major types: reaction of the urticarial, serum-sickness-like type and reactions with an erythematovesicular eruption resembling the trichophytids.

The urticaria and erythemas, together with joint pain, and fever in some instances, comprise the commonest allergic reactions to penicillin. The induced urticarial form of penicillin allergy is often temporary, even transient in character. This is an induced sensitivity and requires a definite incubation period varying from five days to three weeks. In a recent study by Peck *et al.*,<sup>9</sup> it was shown that among 130 patients who received penicillin there were 25, or about 19 per cent, who exhibited such an acquired sensitivity. In approximately 40 per cent of those developing this sensitivity, a positive penicillin test of the delayed type could be elicited. Previous fungus disease is not considered to have played a role in this form of induced sensitivity.

Penicillin reactions of the erythematovesicular type resemble trichophytids. This form of penicillin sensitivity may be conceived of as existing in a latent and active stage. The latent stage is characterized simply by the presence of a positive 48-hour penicillin skin test without any history of previous penicillin administration. Attention to the so-called "spontaneous" positive skin test was first drawn by Welch and Rostenberg. The active stage, based upon the pre-existing latent sensitivity, appears after exposure to penicillin and resembles the trichophytid because it is charac-

terized by an erythemato-vesicular eruption which tends to localize primarily on the hands, feet, and groins but may become generalized.

Among 276 adults who had not received penicillin, there was an incidence of 5.4 per cent with a positive delayed reaction to penicillin.<sup>7</sup> Welch and Rostenberg,<sup>16</sup> in a small series tested, found an incidence of 5 per cent. Sixty-five children below 12 years of age were tested and none showed a positive reaction. Similar observations were reported by Cormia and Lewis.<sup>17</sup>

The work of Peck and Hewitt<sup>14</sup> explains the mechanism by which dermatophytosis may induce a positive penicillin test and ultimately lead to reactions to penicillin which resemble those of the trichophytids. The results of their investigations, as previously cited, showed that the common pathogenic fungi mainly responsible for many of the dermatomycoses are capable of producing an antibiotic possessing many of the properties of penicillin. In the course of a fungus infection of the skin, there are a number of antigens elaborated by the infectious agent. One of those is responsible for the positive trichophytin test and trichophytin sensitivity, and another leads to the so-called "spontaneous" penicillin test and represents the latent phase of penicillin sensitivity.

The relationship of the incidence of the "spontaneous" positive penicillin test to the trichophytin reaction is of interest. The trichophytin test carried out simultaneously in the patients who have not received penicillin treatment showed positive reactions in 33.3 per cent. Among the penicillin-positive patients there were 60 per cent who had showed a positive trichophytin reaction, practically twice as frequent as among penicillin-negative individuals. Furthermore, the percentage of patients sensitive to penicillin among trichophytin-positive individuals was 9.7 per cent, as against 3.2 per cent among trichophytin-negative persons. Additional evidence of the relationship between previous fungus infection of the skin and trichophytin sensitivity on the one hand, and "spontaneous" penicillin sensitivity on the other, is found in our observations on 165 children ranging in age from 2 months to 12 years who had never received penicillin. In none of these was either the penicillin or trichophytin test positive. These negative findings are in accord with the known fact that, even if present, fungus infections in the form of dermatophytosis rarely produce sensitivity to trichophytin in children below 12 years of age.

It has been shown that in spite of practically the same incidence of fungus infection among males and females there was a much higher percentage of males who acquired a sensitivity than females. This trend also applies to penicillin sensitivity. Among 130 patients who received penicillin, there were 32 with skin eruptions, 34.2 per cent of all males developing them as against only 8.3 per cent of females.

Among 276 adults whom we treated who had never received penicillin, there were 168 males, of whom 6.05 per cent showed the so-called "spontaneous" positive reaction. Of 108 females only 4, or 3.7 per cent, showed a positive reaction.

The active stage of penicillin sensitivity occurs during treatment. Unlike

the induced form, this reaction can occur on the first day or two or even on the first administration of penicillin. We had seven such cases. All were males and the trichophytin and penicillin tests were positive in each case. The much higher incidence of positive penicillin and trichophytin tests in this group than in the urticarial form of reaction is indicative of pre-existing sensitivity and its relation to fungus disease. Patients with this type of penicillin sensitivity usually have persistent penicillin allergy of varying degrees.

There is no common antigen between crystallin penicillin and trichophytin. We<sup>18</sup> have been able to show that the antigenicity of penicillin closely parallels antibiotic potency. In contrast with penicillin, trichophytin is much more stable and will act as an antigen after long standing and after heating. As a final evidence that the penicillin and trichophytin antigens are not identical, it is possible, when penicillin and trichophytin sensitivity co-exist, to desensitize the patient with increasing doses of penicillin and convert the positive 48-hour penicillin skin test to negative without in any way altering the trichophytin reaction.<sup>19</sup>

Experimental evidences<sup>18</sup> suggest that streptomycin, as at present commercially available, contains an antigen closely related to penicillin. Penicillin itself may be present in small amounts in streptomycin. Also, there is a possibility that streptomycin and trichophytin contain a common antigen present in larger amounts in the latter. If it is true that there is in streptomycin as it is now available a penicillin-like antigen or even penicillin itself, and an antigen common to both trichophytin and streptomycin, we can expect a high incidence of sensitivity reactions in individuals receiving streptomycin. Furthermore, caution must be carried out in administering streptomycin in patients already sensitive to penicillin.

There are many new antibiotics constantly being reported. Since it is a long and tedious, as well as expensive, process to crystallize these new products, it is perhaps worth while to bear in mind the origin of the antibiotic. If the antibiotic presents an entirely new bacterial spectrum which is different from that in any of the other known antibiotics, then of course there is no question but that a great deal of work should be carried out in obtaining as pure a product as possible. However, if the origin of the antibiotic is from organisms both pathogenic and nonpathogenic, which have the human as the host, it is well to consider that such antibiotics may have a strong tendency to elicit sensitivity phenomenon.

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# PRACTICAL APPLICATIONS OF IMMUNOLOGIC PRINCIPLES IN THE DIAGNOSIS AND TREATMENT OF FUNGUS INFECTIONS

By Donald S. Martin\*

*Department of Preventive Medicine and Public Health, Duke University School of Medicine  
and Duke Hospital, Durham, North Carolina*

The immunologic reactions in most fungus diseases differ markedly from those usually encountered in the acute bacterial, viral, or Rickettsial infections. In general, the fungi causing systemic diseases are relatively non-toxic and weakly antigenic, the immune mechanisms of the body responding only in a sluggish manner to the presence of the invading fungus. However, this weak antibody response may be explained in part by the relative insensitivity of the antigens which we have employed for the cutaneous and serologic tests.

Our experience has been limited largely to cases of North American blastomycosis and pulmonary moniliasis. The test materials used in these diseases have consisted almost entirely of crude saline suspensions of heat-killed fungi. Most of the patients who came to the clinic had had their disease for months or even years and there was no difficulty in establishing the diagnosis by mycologic methods. The immunologic tests, therefore, were applied to obtain immunologic data on individual patients for the purpose of determining the type of therapy. The following data all were obtained by examination of the hospital records of 24 cases of systemic blastomycosis and 20 patients with the cutaneous form of the disease.

With a few exceptions, the skin-testing material used in blastomycosis consisted of a vaccine made from a yeast-like phase of *Blastomyces dermatitidis* cultivated on blood agar at 37°C. The vaccine was standardized by making a 1-1000 suspension of the packed cells in physiologic saline and then sterilized by heating to 60°C. for two hours.

Of the twenty patients with cutaneous blastomycosis, fifteen were skin tested with vaccine and all fifteen reacted positively. Of the twenty-four patients with the systemic type of infection, nineteen were skin tested, but only nine, or about one half, of the patients reacted positively. The remaining ten patients gave negative responses to intracutaneous injections of the vaccine. Of numerous skin tests on patients without blastomycosis, none has given a positive test.

The relative specificity of the skin reaction is in contrast to the experience of others who have tested infected animals and shown cross reactions between *Blastomyces dermatitidis* and *Histoplasma capsulatum* antigens.<sup>1, 2</sup> It should be emphasized that the skin tests on the infected animals were made with "blastomycin" and "histoplasmin," preparations which consist essentially of filtrates of cultures grown for a long time on a broth medium. Such extracts may be more sensitive in eliciting skin reactivity, or nonspecific factors may be contained in the filtrate, accounting for the cross reactions

\* Present address: University of Puerto Rico School of Medicine, San Juan, P. R.

observed in animals. The great variations in potency among different lots of such extracts has been emphasized by Howell.<sup>2</sup> Most probably, the apparent specificity of the reaction to our vaccine is accounted for by the small amount of antigen present in the crude vaccine. Such skin-testing materials probably would be far too insensitive to be of any value in a survey of population groups for evidences of a previous infection, but, from the standpoint of the patient with active disease, the results have proven to be of value. In extremely hypersensitive patients, sterile abscesses are found at the site of injection.

From the standpoint of therapy, a positive skin test in skin blastomycosis is indication for desensitization treatment with vaccine before applying X-ray treatments or administering iodides. Response to small doses of X rays is much more rapid if the patient is partially desensitized, and we have seen skin lesions spread rapidly in a hypersensitive patient who was given large doses of potassium iodide without preliminary vaccine treatment.

The low percentage of positive skin tests to *Blastomyces* vaccine obtained in systemic blastomycosis has been interpreted as indicating a state of anergy in such patients. Of the nine patients with positive skin tests, three died, four showed marked improvement, and two were classified as cured, one of these patients remaining well for the past thirteen years. Of the ten patients with negative skin tests, four have died, two showed no improvement when they left the hospital, and one was not followed. Four patients improved, two of them following treatment with immune serum. In our experience, a negative skin test to the vaccine suggests a more serious prognosis than does a positive reaction.

The present complement fixation tests for *Blastomyces dermatitidis* are unsatisfactory. In most of these tests, we have used as antigen a living suspension of yeast-like organisms. The dose of antigen employed in each test was based on the anticomplementary titer of the suspension, a property which has no relationship to the combining power of the antigen. The large cells of *Blastomyces* in such a suspension provide a relatively low surface to volume ratio and it is not surprising that negative or low titers are obtained in clinical infections. Serologic tests were done on only seven patients with cutaneous blastomycosis, and five of them gave positive results, although the titers were relatively low. Eight of seventeen systemic cases gave positive complement fixation tests, and, in general, the titers were higher in the systemic infection than in patients with only cutaneous involvement.

It was suggested at one time<sup>3</sup> that the prognosis of the patient could be correlated with the reaction to the skin test and the serum antibody titer. However, as more cases have been studied, so much variation has been found that we feel less inclined to attach too much prognostic significance to the results of the tests alone. It is felt that if a more sensitive and better standardized complement-fixing antigen could be developed, the serum antibody titer might have significance.

Preliminary experiments have indicated that a protein fraction, precipitated from a yeastlike *Blastomyces* cell, ruptured by sonic waves, is an excellent complement-fixing antigen. Interestingly enough, the polysaccharide precipitated from these ruptured cells fixes guinea pig complement with im-

mune rabbit sera but not with sera of patients with blastomycosis. This finding corresponds with the well-known results of complement-fixation tests with pneumococcus polysaccharide as antigen, in which fixation is obtained when the rabbit antibody-guinea pig complement-polysaccharide antigen system is used, but not when human antibody is substituted for the rabbit antibody. The extracted protein fractions of *Blastomyces*, however, will fix guinea pig complement with both rabbit and human antibodies.

We have made it a practice to skin test all patients with pulmonary moniliasis. The skin test itself is of no value as a diagnostic agent because of the extremely high incidence of positive skin tests in the general population. *Candida albicans* occurs so frequently in the oral cavities, feces, etc. of normal patients that the finding of a positive skin test in a normal individual should occasion no surprise. It must be emphasized that a diagnosis of pulmonary moniliasis can be made only with difficulty, because *C. albicans* is such a common secondary invader in many types of chronic pulmonary disease. However, once the diagnosis of pulmonary moniliasis has been established, a skin test should be done in order to plan a course of therapy. We have seen hypersensitive patients clear up entirely after injection of desensitizing doses of vaccine. On the other hand, a dramatic improvement followed antiserum therapy in a case of pulmonary moniliasis in which the skin test to vaccine was negative.

Although excellent agglutination reactions are obtained easily with hyperimmune rabbit sera, it has been our experience that the agglutination reactions with human sera are very unsatisfactory even in patients with definite clinical manifestations of moniliasis. The problem was studied by Mrs. Rees in this laboratory, and it was found that clear-cut agglutination occurred with human sera if the yeast cells were washed twice in saline before setting up the agglutination test. That inhibition of the agglutination reaction with unwashed cells was due to the blocking action of soluble antigen in the surrounding fluid was proven by washing the cells and resuspending them in the supernate of the first washing. Such supernates not only reduced the serum titer by one to two tubes but also made a great change in the qualitative appearance of the agglutinated clumps. Agglutination of the washed cells resulted in the formation of definite large clumps which were distinguished easily from the controls.

Unfortunately from the diagnostic standpoint, the increase in sensitivity of the test has resulted in the finding of definite anti-*Candida albicans* agglutinins in a relatively high percentage of normal individuals. In examining 218 sera sent to the serologic laboratory for Wassermann tests, it was found that 65 per cent of these sera gave positive agglutination reactions for *C. albicans* in a titer of 1-20 or higher. Sixty-five of eighty-six females (75 per cent) and seventy-eight of 132 males (57 per cent) gave positive reactions. The highest age and sex incidence (87 per cent) was found in females in the 21 to 40 age group.

The differences in results obtained with unwashed and washed cells were investigated by testing 134 sera using both methods. Of 100 sera which gave negative reactions with unwashed suspensions, 53 were negative also

with the washed cells. Of the remaining 47 sera, 15 gave a titer of 1-20, 20 of 1-40, 8 of 1-80, and 4 of 1-160 with the washed cells.

### *Summary and Conclusions*

It is felt that future efforts should be directed toward the development of more sensitive and better standardized antigens so that the results of serologic and skin tests can be evaluated more accurately and correlated with the clinical picture of the infected patient. Preliminary experiments have indicated that the disintegration of pathogenic fungi by the sonic oscillator may permit the extraction of relatively pure testing substances. It remains to be seen whether or not the development of more sensitive *Blastomyces dermatitidis* antigens will result in the finding of evidence of subclinical infections in endemic areas such as North Carolina.

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## HISTOPLASMIN SKIN TEST

By Alexander M. Iams

*Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota*

In the past five years, histoplasmosis has assumed a more important role in American medicine than most diseases of such apparent clinical rarity. Since Darling's first case of histoplasmosis was reported in 1906, over ninety proven cases of the disease have appeared in the literature.<sup>1</sup> In spite of this small number of reported cases, a great deal of interest has been aroused by a series of papers concerning the histoplasmin skin test and its relationship to pulmonary calcification.

In 1943, Long and Stearns,<sup>2</sup> in a review of 53,400 induction-center roentgenograms of the army inductees, noted that "the incidence of calcified lesions presumed to represent healed tuberculosis corresponds to the now well-known pattern of regional differences in the United States. High incidence, (over 15 per cent) was noted in a region bounded roughly by Fort Oglethorpe, Georgia; Jefferson Barracks, Missouri; Little Rock, Arkansas; and Columbus, Ohio. In general, the calcifications noted in the films of men from this region were considerably larger and more extensive than those in men from other parts of the country. Also, disseminated miliary calcifications variously believed to represent healed residuals of a post-primary hematogenous dissemination of tuberculous, or perhaps in some cases, a healed fungus infection of the lungs, seemed relatively more frequent in this area."

Other authors<sup>3-7</sup> previously noted that, in the area where there was found a high incidence of pulmonary calcifications, there was also a high percentage of negative Mantoux reactors in these same patients.

Smith,<sup>8</sup> in discussing the coccidioidin skin test and its specificity, stated that "some individuals from Missouri, Illinois, Michigan, Indiana, Tennessee, Kentucky, Ohio, West Virginia, Pennsylvania, and New York may have borderline positives, while a few are undoubtedly positive. This is the area of pulmonary calcifications in tuberculous negative persons and is also the endemic area of histoplasmosis."

Some investigators<sup>3-7, 9, 10</sup> feel that the negative tuberculin reactions in patients with pulmonary calcifications are due to loss of sensitivity with the healing of the tuberculous lesion. This has not been generally accepted, however. In 1945, and later in 1946, Palmer,<sup>11, 12</sup> following the suggestions of Smith and of Christie, reported the histoplasmin skin reactions of 10,580 nurses from all sections of the United States. In an area closely simulating that described by Long and Stearns,<sup>2</sup> 68.3 per cent were positive reactors. Christie and Peterson,<sup>13-15</sup> in a series of three articles in 1945 and 1946, reported 2,032 histoplasmin skin reactions, most of which were from the Mississippi River basin. They found percentages of positive reactors ranging from 59.4 per cent to 83.9 per cent. In 1946 and 1947, Zwerling and Palmer<sup>16</sup> reported a series of 698 patients with pulmonary calcification. Of these, 494 were histoplasmin positive, tuberculin negative.

Furcolow *et al.*<sup>17</sup> reported the longest series of more than 17,000 tests from

Kansas City and had findings comparable to those of Christie and Peterson. With all the interest aroused by these observations and because no studies of the histoplasmin skin test in children have been undertaken in Minneapolis, I thought it would be of value to make a random sampling of the children in the Twin Cities. At the present, our study of children in the Minneapolis area is still inadequate to show anything but trends. A more complete survey will be reported later. These tests, compared with a series of tests on adults in Minneapolis being carried out by Johnson,<sup>18</sup> combined with other reported surveys and analyzed as to age, sex, race, tuberculin reaction, and pulmonary calcification, might be of interest.

### *Material and Methods of Present Study*

Through the cooperation of the Department of Pediatrics, University of Minnesota, the histoplasmin skin test was introduced as a routine test on the pediatric floors at the University Hospital and Minneapolis General Hospital. Other studies have been done at the Catholic Boys' Home and Northwestern Hospital, Minneapolis. Tuberculin studies were also done at the same time. Roentgenograms of the chest have been planned for any positive reactors. The antigen used was kindly furnished by Dr. C. W. Emmons of the National Institute of Health. It was from the lot H<sub>3</sub>. The dilution of 1:100 was used and 0.1 cc. was injected intradermally. A test was considered positive if the area of induration was more than 5 mm. in diameter in 48 hours.

So far, we have done 210 tests, of which only two were positive, or 1.0 per cent. This is in contrast to the figures of Johnson<sup>18</sup> of this city who, in 500 adult cases, found 41 (8.0 per cent) positive reactors. His figures agree with those of Palmer.<sup>11</sup> It is also significant that he used a dilution of 1:1000.

Thus, our figures are much lower so far than other investigators, and, if any conclusion can be drawn, it might be said that the histoplasmin sensitivity in this area is developed at a later age than 15 years.

### *Review of Other Studies*

*The Antigen.* Van Pernis, Bensen, and Holinger<sup>19</sup> first prepared histoplasmin from dextrose broth cultures of the fungus. Zarafonitis *et al.*<sup>20</sup> also reported the preparation and use of histoplasmin antigen. However, most of the recent studies have been done with antigens prepared by either C. W. Emmons or Amos Christie. The former prepares<sup>21</sup> his antigen from cultures of *H. capsulatum* grown on a medium similar to that used for tuberculin, containing asparagine, dipotassium phosphate, sodium citrate, magnesium sulfate, ferric citrate, dextrose, glycerine, and water. A three-month culture of the fungus is filtered through a Berkfield N. filter and tested for sterility. Merthiolate is added in concentration of 1:10,000. Christie's method of preparation has not been published, but I assume it is similar to that of Emmons.

*The Test.* The material is injected in dilutions of 1:100 to 1:1000, depending on the investigator; 0.1 cc. of the substance is injected intradermally

and read in 48 and 72 hours. Any test showing an area of induration over 5 mm. is considered positive.

*Specificity.* As with all skin tests used for diagnostic purposes in medicine, there have been questions raised as to the specificity of this test. Van Pernis *et al.*<sup>19</sup> first used the test both on experimental animals and on an established case of histoplasmosis in a human patient. Both gave positive reactions. The most important usage of the test, however, has been in the problem of pulmonary calcification, and, in this, the problem of specificity is of particular importance.

Emmons<sup>21</sup> has reported the first investigation of this matter. He first injected 0.1 cc. of histoplasmin intradermally in dilutions of 1:10, 1:100, and 1:1000 into twelve normal guinea pigs. None of these reacted. In four of these animals, he repeated the tests and in none was there any sign of irritation or sensitization by the antigen. He then tested 39 guinea pigs with experimental histoplasmosis. Thirty-two of these gave reactions to a dilution of 1:100. Also, 9 rabbits, similarly infected, gave positive reactions.

In studying the antigen in relation to other mycoses, Emmons found that, in animals experimentally infected with blastomycosis, coccidioidomycosis, and haplosporangiomyosis, all reacted in variable percentages positively to the histoplasmin antigen. Of particular significance were the reactions to blastomycosis. All 8 test animals were positive reactors. In studying this reaction further, he did titration studies of the antigens of the two fungi, testing their cross reactions in experimental animals in many varying dilutions. From these studies, he concluded that "There seems to be an almost complete cross reaction between histoplasmin and blastomycin in experimental blastomycosis and histoplasmosis in guinea pigs."

In studying 136 hospitalized patients, half of whom had atypical pulmonary lesions, he found 40.4 per cent reacted positively to histoplasmin and 25.7 per cent reacted positively to blastomycin. The histoplasmin test was positive in 97 per cent of those who reacted also to blastomycin, and the blastomycin reacted positively to 61.8 per cent of those histoplasmin positive reactors. From these studies, he concluded that while this test may be of some use in the epidemiological field, its use as a diagnostic procedure was certainly limited.

He also has found cross reactions experimentally with *Candida albicans* and feels this might well explain many of the positive reactors in the reported surveys.<sup>26</sup>

However, Howell<sup>22</sup> has reported the most complete study into the whole subject. Six strains of *Histoplasma capsulatum* and five of *Blastomyces* were investigated. He first compared the reactions of the different lots of each antigen in different dilutions ranging from 1:100 to 1:5000 and found that different lots of histoplasmin and blastomycin gave different percentages of reactors in similar dilutions. From this, he concluded that the number of positive reactors depends on the lot of histoplasmin used and the dilution of the antigen. He recommended, therefore, that the antigens be standardized, preferably by employing a concentration of each lot which would detect a similar percentage of sensitized animals. This concentration he called the titer, which he defined as the minimum amount which would detect sensi-

tivity in approximately 80 to 90 per cent of the sensitive test animals. He had like results with antigens prepared from heat-killed suspension of *H. capsulatum* and *Blastomyces dermatitidis*.

In a different set of test animals, however, he had entirely different percentages of positive reactors. From this, he concluded that the reactions also depend on the stage of the disease at which the tests were done. This he called the level of sensitivity of the animals or patients, and noted its importance in determining the titer of the antigen.

He then tested the cross reactions of the animals between blastomycin, histoplasmin, coccidioidin, and tuberculin. As did Emmons, he found that there was a large number of cross reactions between histoplasmin and blastomycin. However, in using different lots and different concentrations, he found that the percentage of cross reactions depended on the lot and concentration of each antigen. He felt that the number was more apparent than real and that, if the critical titer of each was determined and used, they would be decreased considerably. He felt that the acquirement of blastomycosis did sensitize the animals to the histoplasmin antigen, since a much higher percentage reacted positively to the antigen after acquiring the disease than before. He does not feel that the cross reactions are "almost complete," as Emmons says they are.

Christie<sup>15</sup> has deduced from his studies of the histoplasmin skin test in man that the cross reactions noted by Emmons are not seen in human subjects.

Thus, one sees that the whole field of specificity is wide open still. While the test is not as specific as is coccidioidin, it is probably more specific than Emmons has shown.

### Results

The histoplasmin skin test has been used clinically for diagnostic purposes too few times as yet for any definite information to be gained. It is the general opinion of most investigators, however, that patients with a fully developed clinical picture of the disease are anergic and give a negative reaction. This was my experience with a patient seen at the Mayo Clinic.<sup>1</sup> It was interesting to note that the mother of this patient reacted very positively to the antigen. This has also been the experience of others. The only deduction to be drawn from this is that both patients were exposed to the same source of infection, but of possibly variable dosages. The mother in this case showed no signs of histoplasma infection.

As stated previously, the bulk of the tests reported in the literature have been done in an effort to solve the problem of the patient with pulmonary calcification and a negative tuberculin test. There have been 27,780 tests in all reported in studies of this kind. As shown in TABLE 1, 38.6 per cent (10,732) of these were histoplasmin-positive reactors and 61.4 per cent (17,048) were histoplasmin-negative reactors. None of these patients showed any signs of active histoplasmosis.

In TABLE 2, the reactions of the histoplasmin and tuberculin tests in 18,746 of these patients are compared. In approximately 9,000 other patients, no tuberculin tests were reported. In the former group, 44.13 per cent were

histoplasmin positive and 55.87 per cent were negative, thus closely following those in the larger group. Histoplasmin-positive, tuberculin-positive reactors were only 4.05 per cent, while histoplasmin-positive, tuberculin-negative were 40.56 per cent. Of particular interest is the fact that only 4.36 per cent were histoplasmin-negative, tuberculin-positive, while 51.51 per cent were negative to both tests. Thus, of the whole group, while 44.13 per cent were positive to histoplasmin, only 8.41 per cent were tuberculin reactors. As will be shown later, this percentage is not a true index of the population as a whole, since most of these tests were reported from one section, namely, the central Mississippi River basin. In these present statistics, those tests that were necessarily not included were those of Palmer,<sup>11</sup> which

TABLE 1  
TOTAL HISTOPLASMIN TESTS REPORTED IN LITERATURE

<i>H+ T+</i>	<i>H+ T-</i>	<i>H+ *T?</i>	<i>H- T+</i>	<i>H- T-</i>	<i>H- T?</i>
% 2.8	27.0	8.9	3.0	34.8	23.7
Total No. . . . .	10,732		Total No. . . . .	17,048	
% . . . . .	38.6		% . . . . .	61.4	

Total—27,780

\* Tuberculin results not reported.

TABLE 2  
RELATIONSHIP OF HISTOPLASMIN AND TUBERCULIN TESTS

%	<i>H+</i>	<i>H-</i>	<i>Total</i>
T+	4.05	4.36	8.41
T-	40.56	51.51	91.53
Total	44.13	55.87	100.
Total number of cases . . . . .			18,746

were more general in distribution. His over-all percentage of positive reactors in a group of 8,141 tests was 20.9 per cent. Thus, these present statistics are not comparable. Their importance lies only in the comparison with the tuberculin reactors, which, unfortunately, Palmer did not report.

TABLE 3 shows the histoplasmin-tuberculin reactions of 2,577 (16.1 per cent) patients reported from a group of 16,006 patients studied.<sup>13, 14, 16, 23, 24</sup> All these patients showed varying degrees of pulmonary calcification by roentgenogram. Here, it is interesting to note that 82.5 per cent reacted positively to the histoplasmin test and 18.3 per cent reacted positively to the tuberculin test. Thus, the positive reactors are four times as great as the percentages of Palmer<sup>11</sup> (20.9 per cent) and twice as great as the over-all percentage reported in this paper. The percentage of tuberculin reactors is quite low considering that all these patients showed evidences which heretofore had been considered almost pathognomonic of healed tuberculosis.

*Age.* The majority of these tests reported in the literature have been applied to children. Of the reported 27,780 tests, 62.7 per cent have been in the age limits of birth to 18 years. Furcolow,<sup>17</sup> in reporting 16,000 school children in Kansas City, found 5 per cent positive reactors at 2 years, 20 per cent at 6 years, 30 per cent at 8 years, 50 per cent at 12 years, and 65 per cent at 18 years. Christie<sup>16</sup> reported somewhat higher percentages of positive reactors from Tennessee. He found 18 per cent at 2 years, 36 per cent at 5 years, 58 per cent at 10 years, 80 per cent at 15 years. Olson<sup>26</sup> reported even higher figures from Virginia, but from a much smaller group. He found 47 per cent at 5 years, and 82 per cent at 14 years. These are all in contrast to our own figures of less than 1 per cent in all ages.

As far as development of pulmonary calcification is concerned, all these investigators found the percentages lagging appreciably behind the positive reactors at the same age. This has been explained by the reasoning that it takes quite a bit longer to develop the calcifications than it does the sensitivity and probably it takes several repeated invasions by the organism to cause enough calcification to be seen by X ray.

TABLE 3  
RELATIONSHIP OF HISTOPLASMIN AND TUBERCULIN SKIN TESTS  
TO PULMONARY CALCIFICATION

	<i>H+ T+</i>	<i>H+ T-</i>	<i>H- T+</i>	<i>H- T-</i>	<i>Total</i>
No. ....	335	1791	137	314	2577
% .....	13.0	69.5	5.3	12.1	16.1
Total number of tests. ....					16,006

In the older age groups, the percentages of positive reactors have ranged from 20.9 per cent reported by Palmer<sup>11</sup> to 85 to 90 per cent, reported by Christie<sup>16</sup> and Furcolow.<sup>17</sup>

*Sex and Race.* Sex does not appear to be a factor in histoplasmin sensitivity. However, Furcolow<sup>17</sup> is the only one who has reported this aspect in any detail. More investigation is needed before a definite statement can be made. As far as race is concerned, the white race seems to be more sensitive than the colored race, but this is not enough to be of any significance.

*Location.* Of the 27,780 tests reported, approximately 20,489 (73.7 per cent) have been from the mid-Central states, and most of these from Kansas City or Tennessee. Thus, the percentages may not be an exact index of the whole picture. In the few studies that have included a wider selection, however, the percentages have been about the same. Palmer,<sup>11</sup> as stated before in this paper, found 68.3 per cent of positive reactors in these mid-Central states. The Southwest and California fell far under this percentage. The Southeastern and Northwestern areas showed the lowest percentages.

Christie and Peterson<sup>16</sup> noted a similar distribution, but their percentages were appreciably higher in the outlying states. No studies were made in the Northwest, West, or Southwest, however.

One can see that the areas of highest histoplasmin reaction closely corre-

spond to the areas of tuberculin-negative pulmonary calcifications as described by Long and Stearns.<sup>2</sup> It is from these areas also that the reports of histoplasmin-positive, tuberculin-negative pulmonary calcifications have come. Most of the reported cases of histoplasmosis have also been from these areas.

### Discussion

The present status of the histoplasmin skin test has been presented. So far, a great deal of work has been done and many things learned. A relationship has been found to exist between the sensitivity to histoplasmin and many patients with and without pulmonary calcification.

This sensitivity has been found to develop very early in life in a large percentage of the patients. Much material has been collected to support the theory that the disease is endemic, at least in the Mississippi River basin, and that the great majority of the patients get well without any evidence of the disease or, at most, a varying degree of pulmonary calcification. The test has been found to be of little practical diagnostic value as yet.

Much remains to be learned, however. The work of Howell<sup>22</sup> has shown that the test depends on the lot and dosage of the antigen used and the stage in the disease when the patient is tested. He and Emmons<sup>21</sup> both showed that the result of the test also depends on the general mycotic sensitivity of the patient. That is, a positive reaction does not necessarily mean a sensitivity to histoplasmin. It means that the patient is sensitive to one of several mycotic antigens, particularly blastomycin.

Howell's<sup>22</sup> suggestions for standardization of the antigen and determining the titer of each lot is certainly a step forward. Further clinical titrations with blastomycin are in order. The specificity of the test must be improved before any definite deductions can be made. Further studies on a more generalized nationwide basis must be done.

Emmons<sup>21</sup> reports the isolation of *H. capsulatum* from a hilar lymph node in a child with pulmonary calcification. None were found in 35 other cases examined. More proven cases of this kind are necessary before one can definitely prove that the calcification is caused by histoplasmosis.

Whatever is lacking in the present picture of the specificity of the histoplasmin test, the evidence of its relationship to the tuberculin-negative patient with pulmonary calcification certainly cannot be ignored. This must be more than circumstantial and has opened up again the interest concerning this subject.\*

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\* It has been suggested to me by one of my colleagues in Minneapolis that the problem of Boeck's sarcoidosis be investigated from the point of view of histoplasmosis. I have not been able to find such a study reported as yet.

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# SOME BIOCHEMICAL IMPLICATIONS FROM A STUDY OF GROWTH OF PATHOGENIC FUNGI ON MEDIA CONTAINING SINGLE AMINO ACIDS

By Reginald M. Archibald and Frederick Reiss

*Hospital of the Rockefeller Institute for Medical Research, New York, N. Y., and Department of Biochemistry, School of Hygiene, Johns Hopkins University, Baltimore, Maryland; Department of Dermatology and Syphilology, New York University-Bellevue Medical Center, New York, N. Y.*

The combined efforts of biochemists and mycologists seldom have been brought to bear on a study of the amount and character of growth resulting from utilization of single amino acids by fungi pathogenic to humans. Relatively few studies have been reported which indicate the amino acid requirements of fungi pathogenic for man. Much more has been done on the nutrient requirements of nonpathogenic fungi. Anderson and Emmart<sup>1</sup> studied the relation of certain amino acids to carbon dioxide and mycelium production of *Fusarium oxysporum* and reviewed the significant literature on amino acid metabolism by fungi up to 1934. They observed that, of the amino acids tried, aspartic acid was outstanding in its ability to produce mycelium when added to a basal medium containing glucose and inorganic salts. Mosher *et al.*<sup>2</sup> studied the effect of various minerals, carbohydrates, and amino acids in his work on the nutritional requirements of *Trichophyton interdigitale*. In studying the effect of different amino acids, they omitted one or several amino acids from media which were otherwise fairly complete, and which contained minerals, vitamins, carbohydrate, and all of the other amino acids which were readily available to the workers. Benham<sup>3</sup> reported that addition of asparagine to a medium containing oleic acid, glucose, and inorganic salts increased the yield of *Pityrosporum ovale*. Robbins, Mackinnon, and Ma,<sup>4</sup> in studying the effect of vitamin deficiency on the growth of *Trichophyton discoides*, observed that they failed to improve growth by the addition of many amino acids to a basal medium containing peptone. Robbins and Ma<sup>5</sup> studied in detail the growth requirements of *Trichophyton mentagrophytes* (*T. gypsum*). They added amino acids singly and in combination to a basal medium containing inorganic anions and cations and purified agar. Gottlieb<sup>6</sup> investigated the utilization of amino acids as a source of carbons for the growth of two nonpathogenic fungi. A somewhat similar study had been conducted by Baker and Smith<sup>7</sup> with *Coccidioides immitis*. Robbins and McVeigh<sup>8</sup> observed that hydroxyproline inhibited the growth of 5 pathogenic fungi, including *T. mentagrophytes* (*T. gypsum*) and *T. purpureum*, when asparagine was the only other source of nitrogen in the medium. The inhibition produced by hydroxyproline was largely overcome by the addition of proline but not by the addition of any one of 13 other amino acids.

It has become apparent that, for a study of the effect of different amino acids on growth, the use of fungi (more highly differentiated than bacteria) has several advantages which do not attend the use of bacteria. Because there is more opportunity for morphological variation (there being a greater

variety of structures which can be affected by the nutrients), an effect of individual amino acids on morphology can be observed readily. Consequently, the authors undertook to study some of the effects of individual amino acids on the rate, amount, and character of growth of 13 fungi pathogenic for man.

### Method

The basal media employed contained 4 per cent glucose, 2 per cent (unpurified) Bacto agar, and 0.33 mg. per 100 cc. of each of the following vitamins: thiamin hydrochloride, riboflavin, nicotinamide, calcium *d*-pantothenate, ascorbic acid, and pyridoxine. Sodium phosphate buffer, pH 7.2, was added to give a concentration of 0.016 M.

Fifteen cc. aliquots of basal media were placed in Pyrex test tubes 20 × 250 mm. containing sufficient amino acid (peptide, or peptone), as listed below, to give a concentration of 0.25 per cent of the *l*-form.

ALIPHATIC ACIDS  
*Sulfur-containing*  
*l*-cysteine hydrochloride  
*dl*-methionine  
 glutathione  
*Dicarboxylic acids and amides*  
*l*-glutamic acid  
*l*-glutamine  
*l*-aspartic acid  
*l*-asparagine  
*Monobasic monocarboxylic acids*  
 glycine  
*l*-alanine  
*l*-leucine  
*dl*-isoleucine  
*dl*-valine  
*dl*-serine  
*dl*-threonine

AROMATIC, OTHER CYCLIC, AND OTHER  
 NONCYCLIC STRUCTURES  
*Basic amino acids*  
*l*-arginine  
*l*-lysine  
*l*-histidine  
*Non-basic cyclic acids*  
*l*-tyrosine  
*l*-phenylalanine  
*l*-tryptophane  
*l*-proline  
*l*-hydroxyproline  
 para-aminobenzoic acid  
 casein hydrolysate  
 none  
 peptone (Pfanstiehl)  
 heart extract

The amino acid solutions used to make the media were adjusted to pH 6.8 with 1 *N* sodium hydroxide solution. One tube containing no added amino acid and another with peptone served as controls for comparison.

In addition, the effects of adding an extract of unheated dog or beef heart<sup>9, 10</sup> muscle and of combining 2, or in some cases 3, of certain amino acids were studied.

The phosphate buffer (pH 7.2) was used so as to minimize the effect of the different amino acids on the pH of the final media. Autoclaving at 15 lbs. pressure for 30 minutes lowered the pH to 6.5.

Glutamine solution and the heart extract were sterilized by filtration (Berkefeld)<sup>10</sup> and (when used) were added to the basal media *after* the latter had been autoclaved and cooled to 40° C. and not more than several hours prior to inoculation. Glutamine solution, whether by itself or as present in heart extract, is unstable to heat, especially in the presence of phosphate.<sup>11</sup>

Analysis of these amino acids for carbon, total nitrogen, and  $\alpha$ -NH<sub>2</sub> nitrogen indicated that all except glutamine were of a high degree of purity. The glutamine assayed enzymatically<sup>12</sup> 96 per cent. This was by far the best grade available on the market.

The preparation of casein hydrolysate employed was one which has been reported to have a high content of those peptides which are included by the name "Streptogenin."

Each tube was inoculated with an amount of the respective fungus not exceeding the size of a small pinhead and weighing less than 2 mg.<sup>13</sup> The species studied are *Microsporon audouini*, *Microsporon lanosum*, *Microsporon fulvum*, *Trichophyton purpureum*, *Trichophyton gypseum*, *Epidermophyton inguinale*, *Achorion schoenleini*, *Monilia albicans*, *Blastomyces dermatitides*, *Histoplasma capsulatum*, *Hormodendron pedrosi*, *Sporotrichon schenki*, and *Torula histolytica*.

Tubes were kept at room temperature (23–28°C.). The rate of growth was determined after 7 and 14 days by measuring the height and diameter of the growth above the surface and depth and diameter of the subsurface growth. Note was made of the character of the growth variants such as texture, groove and pigment formation, striking appearance of subsurface growth, appearance of colonies under a Wood's light, and microscopic changes of growth characteristics and variations.

### Results

Photographs of some of the 14-day cultures appear in FIGURES 1 to 11. Detailed measurements of the size of colonies and descriptions of microscopic appearances will be reported elsewhere.

One of the authors has observed<sup>14</sup> that the viewing of colonies under ultra-violet (or violet) light is a distinct aid in differentiating some species of bacteria or of nonpathogenic fungi which show identical cultural characteristics in ordinary light. That is, some species of bacteria and fungi, especially when grown at room or ice box temperature, develop substances which fluoresce strongly under a Wood's light. However, the pathogenic fungi here studied failed to show any striking appearance under the Wood's light.

The nature and amount of growth of *T. gypseum* on the various media closely resembles that reported by Robbins and Ma<sup>5</sup> for *T. mentagrophytes*.

When media containing agar, glucose, vitamins, phosphate buffers, and any *one* of several amino acids are inoculated with pathogenic fungi, some growth occurs. When the only amino acid present is hydroxyproline, the amount of growth is very small, and may appear microscopically to consist of a few filaments extending below the surface of the medium. If, however, the single amino acid present is glutamine, glutamic acid, or arginine, the growth is abundant. When any one of the other amino acids is present, the amount of growth is intermediate between that obtained with glutamine and that obtained with hydroxyproline, and the relative amount of growth obtained depends not only on which amino acid is present but also on the species of fungi used for inoculation.

Although fungi grown on media containing only one amino acid have not been hydrolyzed and analyzed for amino acids, they may be expected to contain proteins as postulated by Robbins and Ma.<sup>5</sup> These proteins contain at least several and probably many amino acids. Because of lack of actual analysis of hydrolysates of the growths, caution must be exercised in drawing

conclusions. However, the implication is that the pathogenic fungi studied can use the amino nitrogen of any one of several amino acids to synthesize other amino acids necessary as building blocks in the formation of the protoplasmic proteins characteristic of the species with which the media are inoculated. Any one of these organisms is capable of synthesizing proteins neces-

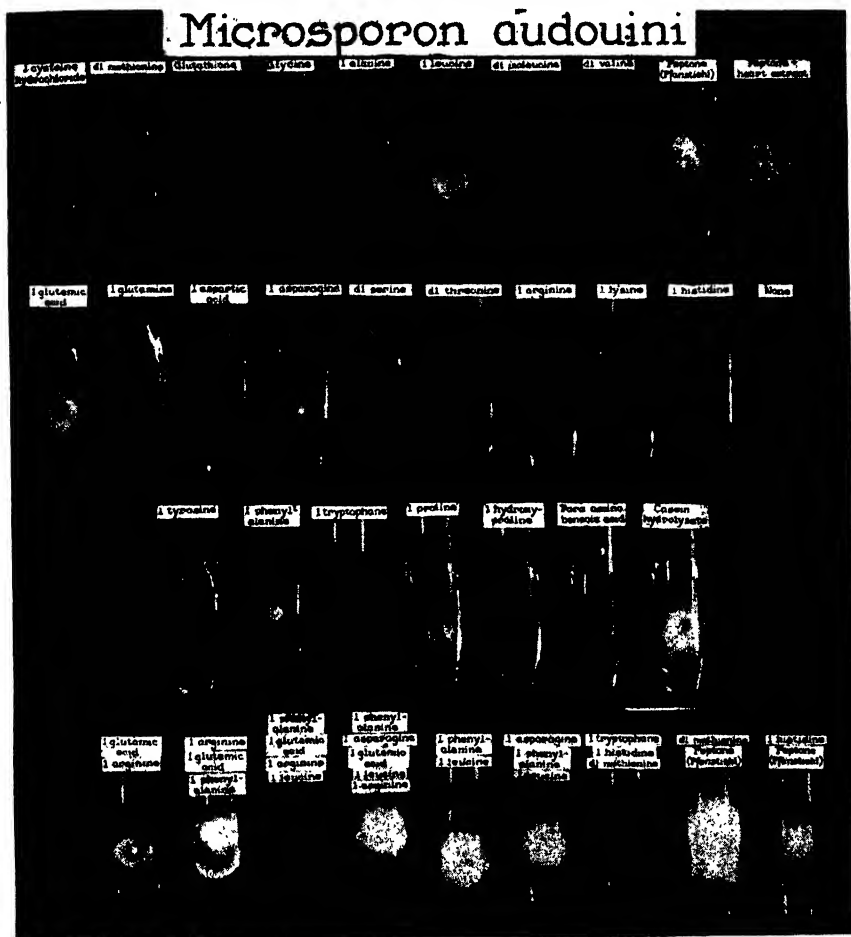


FIGURE 1. Colonies of *Microsporon audouinii* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s) as a source of nitrogen. Reflected light.

sary for its growth and cell structure from any one of several amino acids. For example, the colony which grew on the media containing no amino acid other than arginine contains, presumably, a protein made up of peptide linkages between several kinds of amino acids in addition to arginine. That is, given one amino acid, these organisms can synthesize other amino acids from it. This ability to synthesize other amino acids appears to be greatest



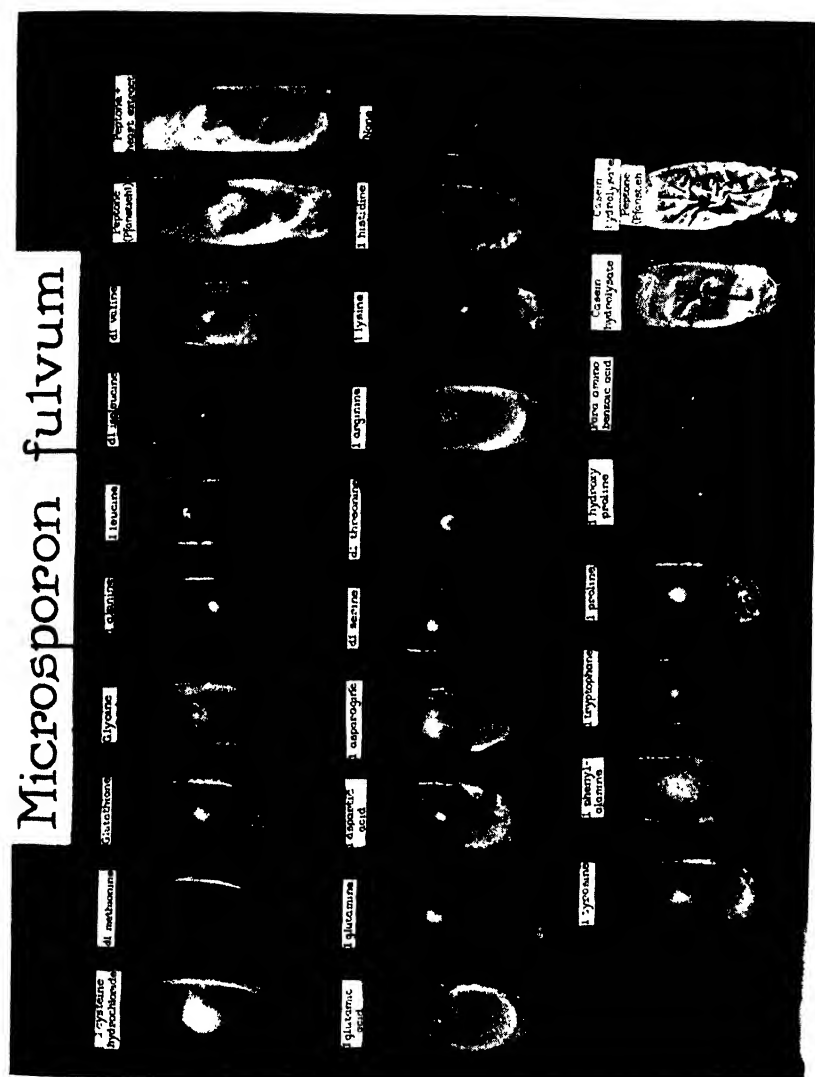


FIGURE 3. Colonies of *M. rosabaron furum* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Reflected light.

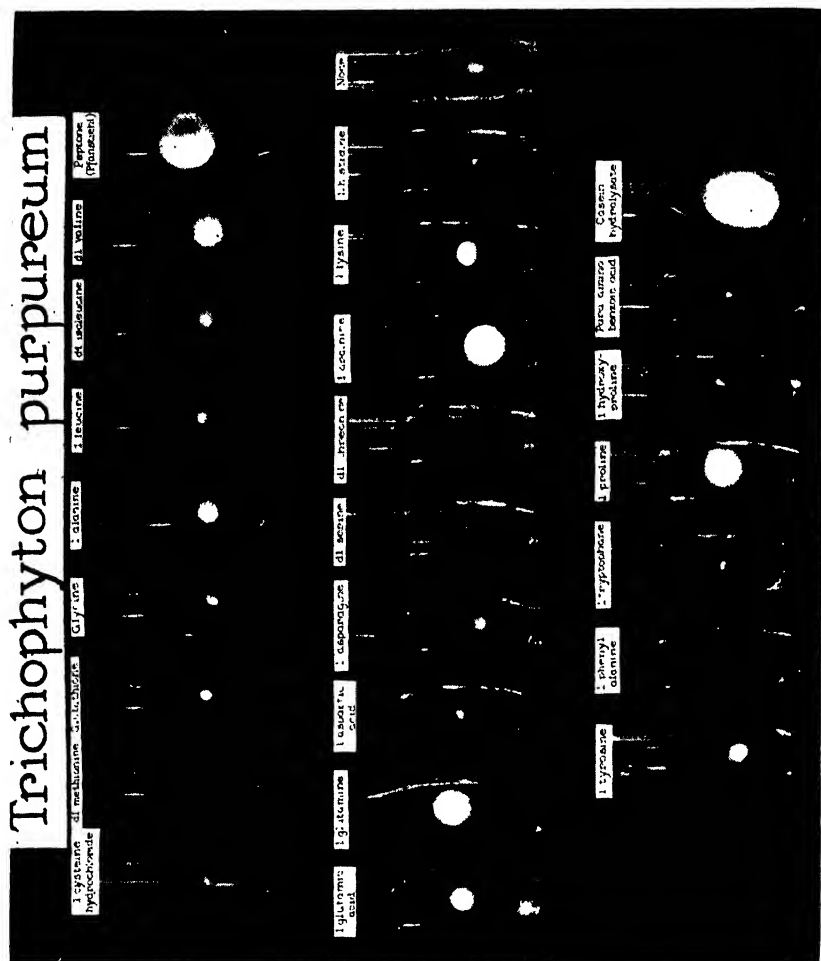


FIGURE 4. Colonies of *Trichophyton purpureum* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Reflected light

when the nutrient amino acid is either glutamine, arginine, or glutamic acid. However, leucine, asparagine, proline, and phenylalanine likewise appear capable of acting as precursors for other amino acids. This is true to a much smaller extent of hydroxyproline, para-aminobenzoic acid, tryptophane, lysine, and methionine. Even the simplest amino acid, glycine, gives good growth with *Microsporon lanosum* and moderately good growth with *Microsporon fulvum*, although very poor growth with *Microsporon audouinii*.

The only amino acids which, when present singly, support fairly good growth of *Achorion schoenleini* are arginine and leucine.

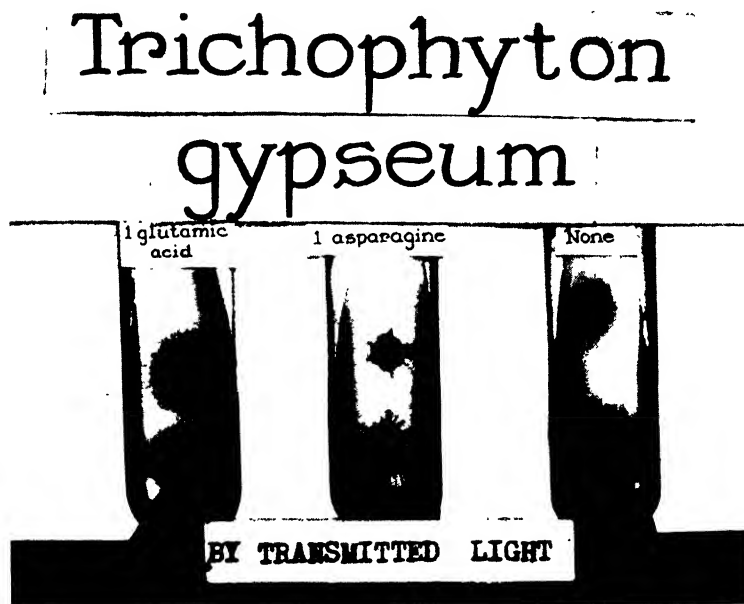


FIGURE 5. Colonies of *Trichophyton gypseum* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Transmitted light.

Slight differences in structure of the amino acid cause great differences in their ability to support growth of fungi. For example, growth of all species tested is better on proline than on hydroxyproline—better on leucine than on isoleucine.

Certain amino acids seem to favor the development of certain types of growth. The cultural characteristics of the colonies are in many cases determined not only by the species used but also by the amino acid present. Thus, a very pronounced coarse arborization of subsurface growth is noticed with *Trichophyton gypseum* (see FIGURE 5), most marked when the amino acid supplied is asparagine.

#### Discussion

It will be observed that the list of several amino acids which can produce good growth has no apparent relationship to the list of amino acids which are



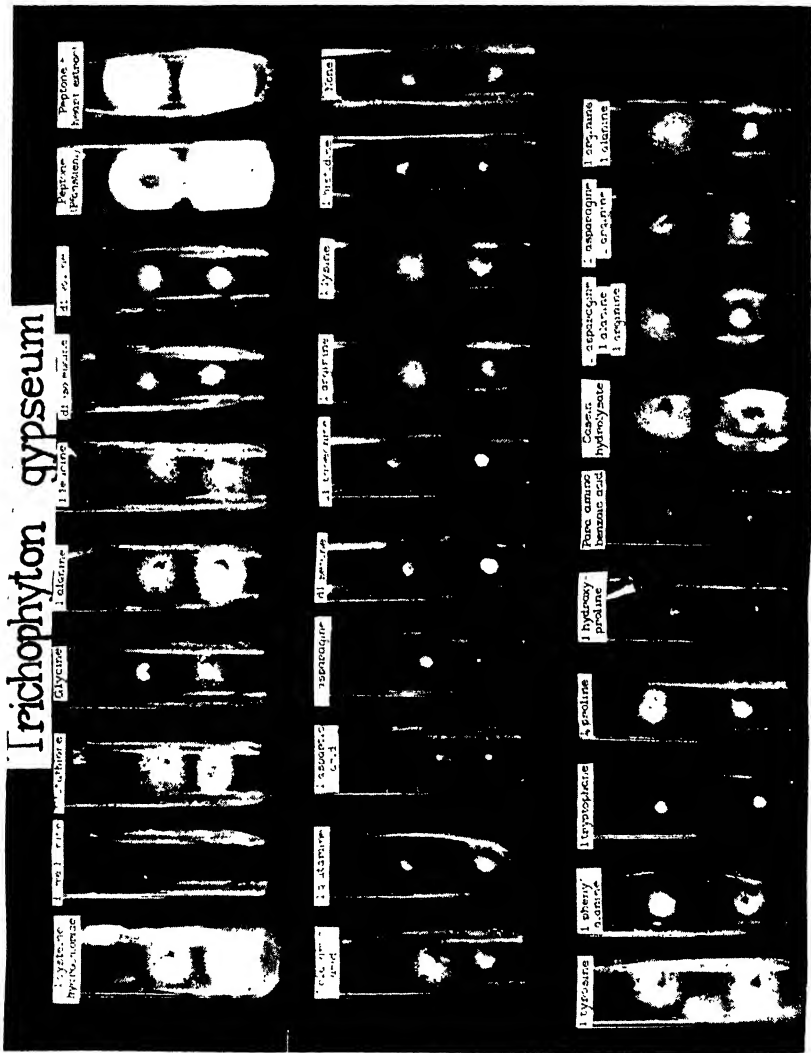


FIG. 1. Colonies of *Trichophyton gypseum* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Reflected light.

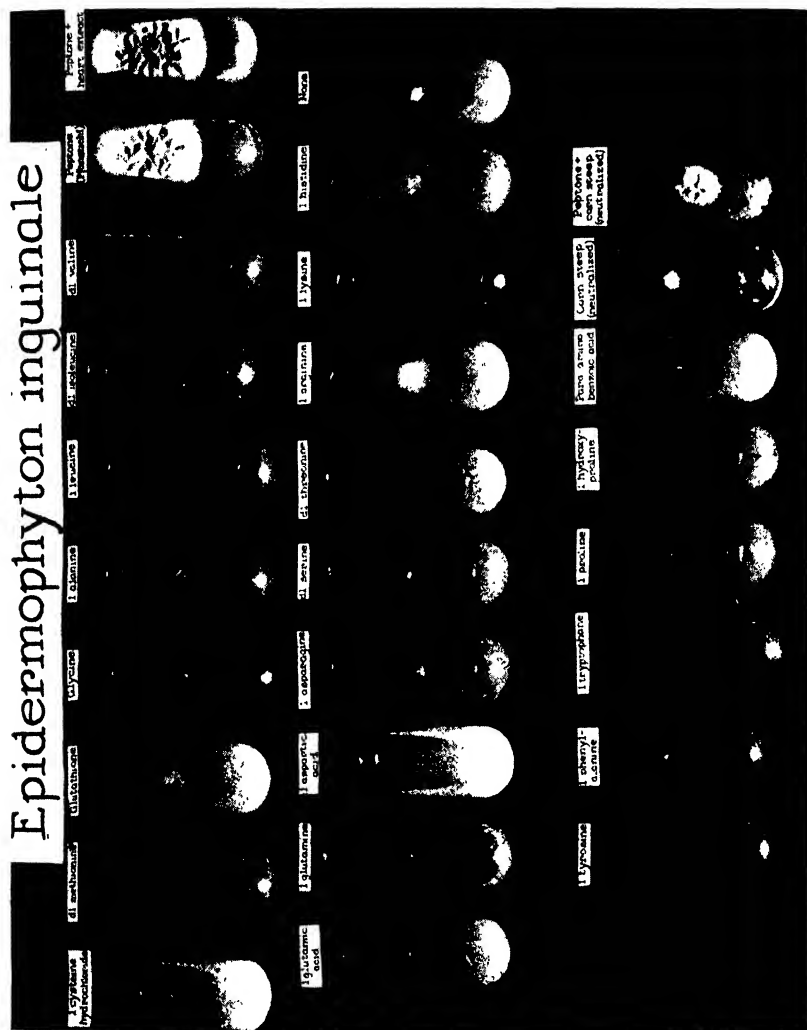


FIGURE 7. Colonies of *Epidermophyton inguinale* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Reflected light.

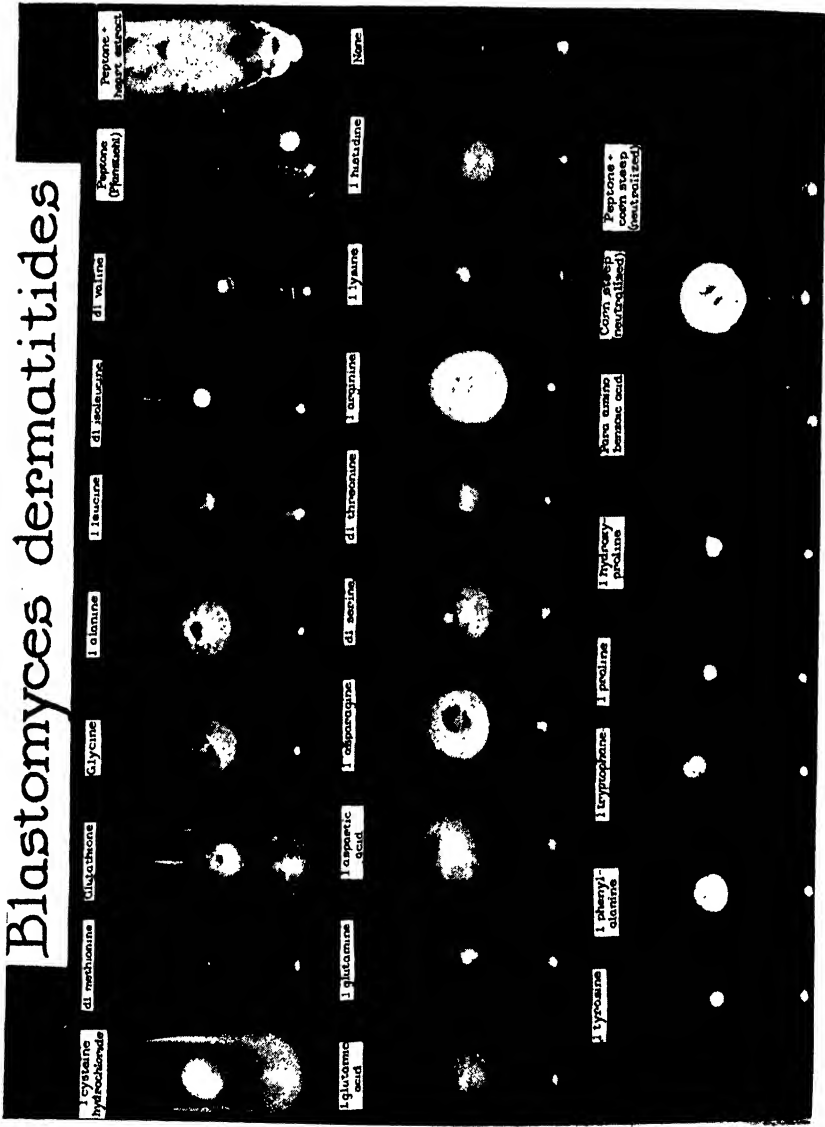


FIGURE 8. Colonies of *Blastomyces dermatitidis* grown for 2 weeks on media containing the indicated amino acid or peptides. Reflected light.





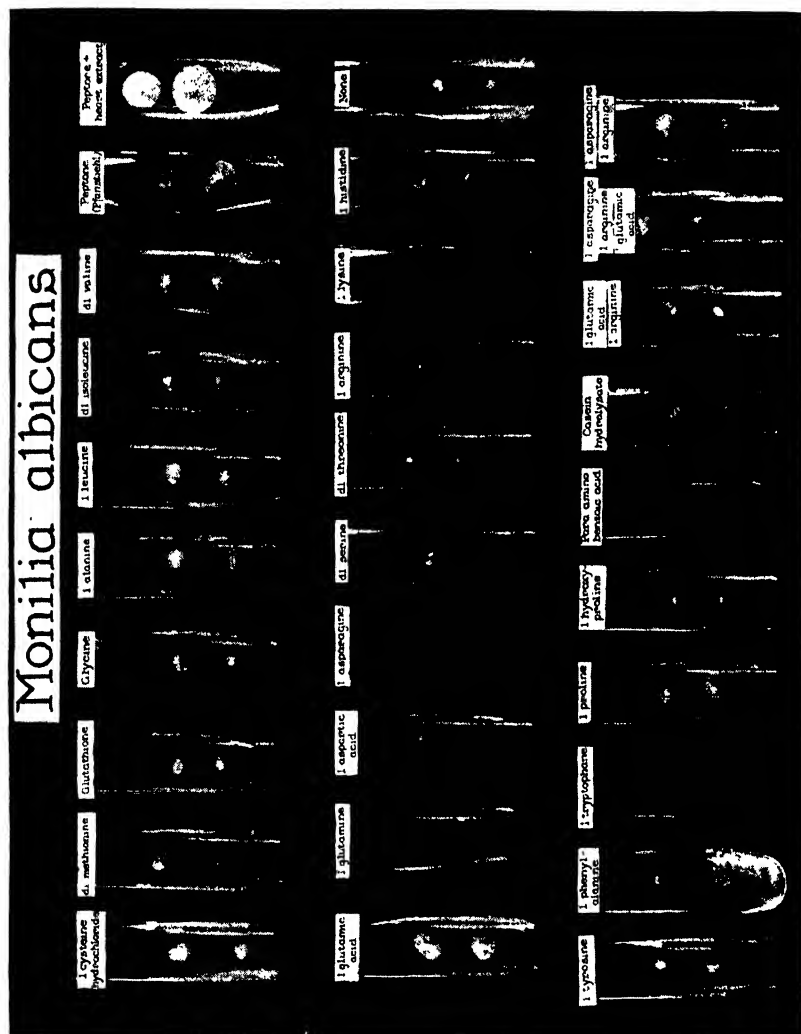


FIGURE 11. Colonies of 2 strains of *Monilia albicans* grown for 2 weeks on media containing the indicated amino acid(s) or peptide(s). Reflected light.

essential for mammalian growth. Rather, it appears more closely related to the facility with which its members can participate in reactions involving transamination. The ability of pathogenic fungi to synthesize many amino acids from a single amino acid should not be surprising in view of the much earlier findings of plant physiologists who studied conversion of seed proteins to glutamine and asparagine and the reconversion of this glutamine and asparagine to proteins built into the growing seedlings.

There is considerable data in the literature which indicates that non-pathogenic fungi and yeasts are likewise capable of synthesizing numerous amino acids from a single amino acid. Kraut and Schlottmann<sup>16</sup> analyzed yeast protein, indicating its percentage composition of histidine, lysine, cystein, tryptophane, and tyrosine. Block and Bolling<sup>16</sup> indicate the amount of ten essential amino acids present in yeast powder. Klose and Fevold<sup>17</sup> (cf. also<sup>18</sup>) grew *Torulopsis utilis* and *Saccharomyces cerevisiae* on molasses (which is chiefly a carbohydrate medium) and found that the protein in the yeast so grown, although somewhat deficient in methionine, apparently was otherwise a fairly complete protein. Tumara<sup>19</sup> showed that a *Mycobacterium lacticola*, growing on a medium containing, as the only nitrogen sources, asparagine and ammonium lactate, synthesized arginine, histidine, lysine, phenylalanine, proline, valine, and tryptophane as well as other amino acids. Abderhalden and Rona<sup>20</sup> observed that *Aspergillus niger* synthesized glycine, alanine, leucine, glutamic acid, and aspartic acid as they grew when the media contained any one of either potassium nitrate, glycine, or glutamic acid as the nitrogen source. Vorbrodt<sup>21</sup> isolated tyrosine, leucine, and alanine from the protein synthesized by *Aspergillus niger* growing on a medium containing only inorganic nitrogen. Skinner,<sup>22</sup> as a result of feeding tests on rats and crude colorimetric tests, concluded that the proteins synthesized by *Aspergillus niger*, *Trichoderma konigi*, *Zygorrhynchus moelleri*, *Aspergillus oryzae*, *Aspergillus terreus*, and *Penicillium flavo-glaucum* contained all of the so-called essential amino acids even when grown on medium containing nitrogen only in the inorganic form. A somewhat similar observation has been made by Takata<sup>23</sup> with *Aspergillus oryzae*.

In general, it may be said that *Hormodendron pedrosi* and the yeast-like organisms studied, *Monilia* and *Torula*, do not have as exacting amino acid requirements as the other species. They grow fairly well when any one amino acid is present.

Williams<sup>24, 25</sup> has conducted numerous experiments with a wide variety of fungi and observed the amount and nature of the subsurface growth obtained when his medium contained hydrolyzed hair or skin, or cysteine, or when high oxygen tension was maintained. Williams observed that abundant subsurface growth occurred not only when cysteine was the amino acid present but also when it was substituted for by other amino acids.

Our work appears to indicate that subsurface growth is abundant when the medium is inadequate for optimum growth. The greater the number of amino acids present, especially if these amino acids are capable of supporting good growth, the smaller is the amount of subsurface growth obtained. The subsurface growth appears, then, to represent an attempt on the part of the

organism to reach out for nutrient of better quality or greater variety than is present near the surface.

Because the agar was not purified and contained some nitrogen, it might be argued that this nitrogen supported some growth. Furthermore, some may say that the inoculum contained some amino acid or protein which influenced the amount of growth. However, because of the very small amount of growth found in the control tube to which no amino acid was added, it can be concluded that the effect of amino acid from the inoculum and agar was almost negligible.

Most of those amino acids which fail to support growth should not be regarded as inhibitors. Rather, it may be assumed that the organisms are not able to convert (at sufficient rate) such amino acids to any other amino acid which in turn could act as a precursor of all of the acids necessary for building the proteins required for growth. For example, as seen in FIGURE 1, *M. audouini* fails to grow on medium containing histidine, methionine, or tryptophane either singly or combined. If, however, histidine is added to an adequate medium containing peptone, the growth obtained is as good as if histidine were absent. Addition of methionine to peptone even slightly increases the growth obtained.

The inhibition of growth (of *T. mentagrophytes*, etc.) noted by Robbins *et al.*, when hydroxyproline was added to media containing asparagine without other amino acids, is interesting. That this inhibition is overcome by proline is not surprising. It appears likely that hydroxyproline competes with proline for the same enzyme systems which convert these amino acids to other amino acids. The hydroxyproline is less readily converted and so may act as a competitive inhibitor. Because other amino acids are not as closely related structurally as these two, they do not compete to the same extent. However, if the medium is adequate with respect to all amino acids, the need for action of these enzymes which convert one amino acid to another disappears, and growth then would be expected to be less inhibited by the presence of hydroxyproline.

### Summary

Thirteen species of fungi (or yeast) pathogenic for man have been grown on media containing carbohydrate, minerals, vitamins, and any one of several amino acids. Most of the amino acids when present singly are able to support growth and therefore are assumed to be transformed into all of the other amino acids necessary for building cytoplasmic proteins, as postulated by Robbins *et al.* for *T. mentagrophytes* and by many others for nonpathogenic fungi and yeasts. The amount and character of growth depends not only on the species used for inoculation but also on which amino acid is supplied as a source of nitrogen. The amount of subsurface growth appears in general to be inversely proportional to the ability of the amino acid supplied to support growth. The more adequate the medium, the less deeply do the subsurface filaments penetrate. Those amino acids which most readily support growth are not necessarily ones regarded as essential for mammalian growth. Hydroxyproline, lysine, tryptophane, histidine, and methionine support growth



very poorly. The yeast-like organisms studied and *Hormodendron pedrosi* are less fastidious in amino nitrogen requirements than the other organisms.

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